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From: Hunt, Jennifer
Sent: Saturday, July 28, 2001 1:48 PM
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Please send me the following ASAP:

Oncologist, (1997) 2/5 (284-299)

Proc Annu Meet Am Assoc Cancer Res, (1996). Vol. 37, pp. A3262

Proc Annu Meet Am Soc Clin Oncol, (1996). Vol. 15, pp. A1811

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Melanoma Res, (1993). Vol. 3, pp. 51

Cancer Immunol Immunother, (1977). Vol. 2, No. 1, pp. 27-39

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CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY, (1997 Dec) 85 (3) 265-72

Proc Annu Meet Am Assoc Cancer Res, (1996). Vol. 37, pp. A3229

CANCER IMMUNOLOGY, IMMUNOTHERAPY, (1996 Nov) 43 (3) 174-9

CANCER RESEARCH, (1991 May 15) 51 (10) 2731-4

Melanoma Research, (1995) Vol. 5, No. 6, pp. 443-444

Thanks,

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was greater than that of uncoated cells in the presence of equivalent concentration of IL-2. Such cytokine-antibody fusion proteins may prove useful in targeting the biological effect of IL-2 to tumor cells.

#3225 Monday, April 22, 1996, 08:25–08:40, Room 10

Therapy of poorly immunogenic murine tumors with tumor peptide-pulsed dendritic cells: induction of a specific antitumor immune response mediated by T cells, Th1-associated cytokines and B7 costimulation. Zitvogel, L., Mayordomo, J.I., Tjandrawan, T., DeLeo, A.B., Lotze, M.T., Storkus, W.J. *Univ. Pittsburgh, Pittsburgh. Cancer Institute, Pittsburgh, PA 15261.*

Dendritic cells (DC) are arguably the most potent antigen presenting cells to prime naïve T cells *in vitro* and *in vivo*. We have previously demonstrated that bone-marrow (BM)-derived DC pulsed *ex vivo* with synthetic tumor-peptides serve as potent antitumor vaccines efficient to protect against immunogenic tumors. To test this approach in the treatment of non immunogenic tumors expressing as yet uncharacterized epitopes, syngeneic GM-CSF+IL-4-stimulated BM-DC, pulsed with unfractionated acid-eluted tumor peptides were injected in three i.v. injections of 5×10^5 cells in tumor bearing mice. The growth of a day 4–8 established sarcoma MCA205 ($H\text{-}2K^b$) or mammary adenocarcinoma TS/A ($H\text{-}2K^d$) were dramatically suppressed with complete tumor eradication in the C3 model (HPV16- transfected fibrosarcoma). The DC-mediated antitumor immune response was dependent on CD4+ and CD8+ T cells and transfer of spleens from immunized mice could completely protect sublethally irradiated naïve animals against a subsequent tumor challenge. IL-12 was critical to initiate the DC-induced immune response since depletion of mice with mAb anti-p40-IL-12 abrogated the DC-induced antitumor effects, whereas depletion with mAb anti-IL-4 did not. Similarly, inoculation of the chimeric fusion protein CTLA4-Ig inhibited the therapeutic effects of peptide-pulsed DC *in vivo*. Therefore, we reported (*J. Exp. Med., in press*) that DC pulsed with MHC class I and/or class II-eluted tumor peptides could be a valuable specific active immunotherapy of cancer.

#3226 Monday, April 22, 1996, 1:00–5:00, Poster Section 5

***In vitro* immunisation of peripheral autologous T cells by tumor extracts processed by autologous macrophages.** Coulon, V., Ravaud, A., Huet, S., Verdier, D., Gualde, N. *Institut Bergonié, Comprehensive Cancer Center, Bordeaux, France.*

The aim of the study was to assess the capability of macrophages issued from bone marrow stem cells to process tumor cells antigens and then to interact with lymphocytes. Macrophages from patients with melanoma were obtained *in vitro* by culturing, for 4 weeks, bone marrow stem cells in the presence of both GM-CSF and SCF (AACR 1994, abs #3081). These macrophages express the CD14, CD33, CD64 and CD71 markers; they produce cytokines and can *in vitro* kill various tumor cell lines (AACR 1995, abs #2810). Melanoma tumor cells were cultured for 4 weeks. Lymphocytes were obtained either from the tumor itself or from the blood. Tumor cells were freeze-thawed and combined with macro-phages activated by INFγ and LPS (ratio 1:1). Macrophages were then cocultured with autologous lymphocytes for 4 to 5 days. Lymphocytes response was evaluated both by assessment of thymidine uptake and generation of cytotoxic T cells killing autologous tumor cells. Under these conditions the proliferative response of T cells was three times higher than controls and the killing was enhanced as well. In some experiments we noticed a suppressive effect. It is likely that bone marrow issued macrophages could be used as antigen presenting cells for active anti-melanoma immunization.

#3227 Tuesday, April 23, 1996, 1:00–5:00, Poster Section 10

Anti-tumor activity and immune responses induced by human cancer apomucin. Yuan Mei, Ma YunGuo, Fei LiHua, Li Li. *Cancer Res. Lab. General Hospital of PLA Beijing 100853 China.*

Mucin molecules are displayed on most human cancer cell surface, and are different from that expressed on normal cells. Some molecular structure of apomucin were identified recently. However, the function of apomucin is only poorly understood. To further elucidating the role of apomucin in the modulation of cancers, this study was to isolate and characterize the apomucin to investigate the immune responses induced. The cancer-associated mucin was isolated from pancreatic cancer cell line SW1990 by chromatography and CsCl density gradient centrifugation. The isolated mucin has a high content of carbohydrate (85%), high molecular weight (670KD). After treated with anhydrous HF to remove the carbohydrate. This apomucin was a mixture of peptides with molecular weight from 28–>90KD, including MUC-1, MUC-2 and MUC-3 verified by ELISA and SDS-PAGE, Western blot. When immunized with this apomucin plus Detox, all of nine mice appeared delayed type hypersensitivity, as shown by mearsing the footpad swelling following intradermal injection of apomucin or synthetic peptide MUC2 or MUC3. The splenic cells of vaccinated mice were cocultured with apomucin 40ug/ml and rhIL-2 50u/ml in vitro. The proliferative lymphocytes elicit cytotoxicity against cancer cells expressed epitope MUC1 or MUC2 or MUC3, measured by ^{51}Cr release assay. These toxicity can be blocked by antibodies against MUC2 or MUC3. Lymphocytes from tumor draining lymph nodes of patients with gastric cancer, colonic cancer and breast cancer were also proliferated when cocultured with apomucin 50ug/ml and IL-2 50u/ml in vitro, the expended cells developed strong cytotoxicity against cancer cell lines: Kato-3, Ls174t and SW1990. These results provide the rationale basis for the use of apomucin as a tumor vaccine to stimulate anti-tumor immunity.

#3228 Sunday, April 21, 1996, 8:00–12:00, Room 15

Induction of melanoma reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A *0201 binding residues. Parkhurst, M., Salgaller, M., Southwood, S.*, Robbins, P., Sette, A.*, Rosenberg, S.A., Kawakami, Y. *Surgery Branch, National Cancer Institute, NIH, Bethesda, MD 20892 and *Dept. of Immunology Cytel Corporation, San Diego, CA 92121.*

Recognition of the melanoma antigen gp100 by tumor infiltrating lymphocytes (TIL) *in vitro* has been correlated with tumor regression in patients with metastatic melanoma treated with the adoptive transfer of TIL plus IL-2. Three common gp100 epitopes have been identified which are recognized in the context of HLA-A2 by TIL from different patients: $G9_{154}$ (KTWGQYWQV), $G9_{209}$ (ITDQVPFSV), and $G9_{280}$ (YLEPGPVTA). To attempt to enhance the immunogenicity of these peptides, 53 synthetic peptides containing amino acid substitutions at HLA-A*0201 binding anchor positions, but not at TCR contact residues, were screened. Several modified gp100 peptides bound with greater affinity to HLA-A*0201 than unmodified peptides and were recognized by TIL specific for the natural epitopes. These peptides were used to sensitize PBL from HLA-A2+ melanoma patients *in vitro*. After 5 weekly restimulations with either the native $G9_{209}$ or $G9_{280}$ peptide, melanoma reactive CTL could only be induced from two of 7 patients. However, amino acid substitutions in these peptides enabled the induction of melanoma reactive CTL from all 7 patients. These results suggest that selected modified gp100 peptides with enhanced *in vitro* immunogenicity may also be more immunogenic *in vivo* and may therefore be useful in immunotherapy protocols for patients with melanoma.

#3229 Tuesday, April 23, 1996, 1:00–5:00, Poster Section 9

Oligoclonal T-cell response in metastases of melanoma patients responding to an hapten-modified tumor cell vaccine. Sensi, M., Farina, C., Maccalli, C., Lupetti, R., Nicolini G., Anichini, A., Berd, D.*, Parmiani G. *Istituto Nazionale Tumori, Milano, Italy and *Jefferson Medical College, Philadelphia, PA.*

Metastatic melanoma patients treated with an autologous, dinitrophenyl (DNP)-modified cell vaccine develop an inflammatory response at tumor sites. T-cell receptor β chain variable (TCRBV) gene usage in T-cells infiltrating post-vaccine metastases from 6 patients was analysed by PCR and compared with that of pre-vaccine lesions and PBL. Few TCRBV regions were overexpressed in most post-vaccine lesions including, in 3/6 patients, TCRBV14. Sequencing indicated the presence of dominant TCRBV14 transcripts, identical when asynchronous metastases of a single patient (Pt 2) were considered. TCRBV14+ T-cell lines obtained from Pt 2 multiple lesions mediated HLA-restricted autologous tumor lysis. These data indicate that, following DNP-vaccination, in vivo expansion of anti-tumor T-cells with a preferential usage of TCRBV families occurs. Partially supported by AIRC, Milan.

#3230 Wednesday, April 24, 1996, 8:00–12:00, Room 20

Eradication of experimental metastases by vaccination with interleukin-12-transduced carcinoma cells. Rodolfo, M., Zilocchi, C., Cappetti, B., Parmiani, G., Colombo, M.P. *Istituto Nazionale Tumori, Milano, Italy.*

Vaccination with C26 colon carcinoma cells engineered to produce IL-12 (C26/IL-12) resulted in the cure of 40% of mice bearing lung metastases of the C51 colon carcinoma, which shares tumor associated antigens (TAA) with C26. Vaccination with C26 producing IL-2 (C26/IL-2) reduced the number of metastases without affecting survival. Anti-TAA C-fixing antibodies were induced by vaccination with C26/IL-12 but not with C26/IL-2. Both treatments induced anti-TAA CTL in the lymph nodes draining the vaccination site and in the spleen. After treatment, T cells from draining lymph nodes and from lungs produced IFNγ and IL-4 with a kinetics which depended on the cells used as vaccine. These results indicate that the better therapeutic efficacy of vaccination with IL-12 secreting tumor cells resulted from the combination of anti-TAA CTL response, production of C-binding anti-TAA antibodies, a faster activation of IFNγ producing T cells in the lungs and a lower activation of IL-4-producing T lymphocytes. Partially supported by AIRC, Milan.

#3231 Monday, April 22, 1996, 10:55–11:10, Room 10

Proliferation of T-cells from colon cancer patients by peptides based on the structure of an anti-idiotype antibody mimicking CEA. S. Pervin[1], A. Sherratt[1], H-T. Wang[1], E. J. Blalock[2], M. Bhattacharya-Chatterjee[1], H. Kohler[1], K.A. Foon[1] and S. K. Chatterjee[1]. *[1]Markey Cancer Center, Univ. of Kentucky, Lexington, KY-40536, and [2]Univ. of Alabama, Birmingham, AL-35294.*

We developed an anti-idiotype antibody, 3H1, mimicking CEA for therapy of cancer patients. By comparison of the amino acid sequences of the complementarity determining regions (CDRs) of 3H1 with CEA, we found that the light chain CDR-2 of 3H1 showed the maximum homology with CEA. By using a computer algorithm based on the molecular recognition theory, this region of 3H1 was also identified as the one involved in idiotype-anti-idiotype contact. Two peptides were synthesized based on the amino acid sequence of this region to examine their potential as T cell epitopes in colorectal cancer patients. Peripheral blood mononuclear cells (PBMC) from 5 of these patients were used for cell proliferation assay before and after 3H1 therapy. PBMC from 2/5 patients were stimulated by both of these peptides multiple times during the course

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in the literature the concept of low and high grade melanoma.[3] We studied the disease-free interval of 181 patients from our data base presenting with a Breslow thickness of the primary lesion in the extremes of the prognostic spectrum; 52 patients with a thickness of 10 mm or greater and 129 patients with 1 mm or less. Nearly 40% of patients with a thick lesion had metastases at presentation and all developed them within 3 years. In contrast nearly 40% of patients with lesions measuring 1 mm or less survived disease-free beyond 10 years, but were at risk of a recurrence as late as 20 years after initial diagnosis. We present representative cases treated systemically for thick, high risk primary lesions. Preliminary observations on 23 patients with thick, high risk primary melanoma and metastases to regional lymph nodes, treated with pre-operative vindesine and DTIC (protochemotherapy) will be discussed.

1. Retsas S. On the antiquity of cancer, from Hippocrates to Galen. In: Retsas S, ed. *Palaeo-oncology: The Antiquity of Cancer.* London: Farrand Press, 1986; 41–58.
2. Retsas S, Gershuny A. Central nervous system involvement in malignant melanoma. *Cancer* 1988; **61**: 1926–1934.
3. Retsas S. Early or low-grade melanoma? *Lancet* 1987; 276–277.

6

Melanoma: a dermatologist's dilemma

C. Fuller, F. Child, E. Higgins and A. du Vivier

King's College Hospital, London, UK

The management of thin melanomas is straightforward since primary excision is usually curative. The current dilemma facing the clinical dermatologist is what to offer those patients in the intermediate and thick lesion groups who have a more guarded prognosis. Our policy prior to 1989 was to excise the melanoma and then follow an expectant policy referring only if and when metastases, diagnosed clinically, occurred. In the last 5 years we have altered our approach and referred all patients with thick melanomas to an oncology department immediately after surgery. We report on eight patients (four male and four female), median Breslow thickness 4.38 mm (range 3.2–21 mm). All had elaborate staging procedures which were pronounced negative. An expectant policy was thus adopted by the referral centre. Six out of eight subsequently relapsed (one local only, four lymphatic only and one local and lymphatic metastases) at a mean of 28.4 (1–68) weeks from negative staging; all patients with secondaries subsequently received conventional therapy and remain alive. In contrast, we have seen 119 patients between 1989 and 1994 with thin lesions who we did not refer. Only one has so far metastasized. With the advent of more successful therapeutic modalities, we ask whether high risk patients should be offered adjuvant therapy and if an open trial should be designed?

7

Surgical adjuvant treatment of melanoma: levamisole and isoprinosine

L. E. Spitler

Northern California Melanoma Centre, San Francisco, CA, USA

We conducted a prospective randomized trial of levamisole *vs* placebo as surgical adjuvant treatment in 203 patients with malignant melanoma who were at high risk for recurrence.[1] Of the patients randomized, 104 received levamisole and 99 received placebo. There was no difference between the treatment and control groups with regard to any of the three end-points analysed. These included disease-free interval, time to appearance of visceral metastases and survival. Moreover, there was no significant difference between the treatment and control groups after adjusting for age, sex or stage of disease. These results differ from the results reported by Quirt *et al.*[2] who reported benefit in the group of patients randomized to receive levamisole. This difference could be due to the slightly different dose of levamisole used in the two studies, or could be due to other factors, such as the fact that control patients in the study reported by Quirt did not receive placebo. In a follow-on study, not yet published, we conducted a randomized double-blind trial of isoprinosine *vs* placebo as adjuvant therapy for surgical treatment in 280 patients with melanoma. Of the patients entered into the study, 143 received placebo and 137 isoprinosine. The distribution of important prognostic variables was similar in both groups, indicating the efficacy of the randomization and the absence of bias. No serious or life-threatening side effects were noted. Elevation of serum uric acid levels were noted in some of the patients randomized to isoprinosine. There was no statistically significant difference between the treatment and control groups regarding any of the three end-points. There was a trend in favour of the isoprinosine group in patients receiving 3 months of treatment and in patients with stage I disease, but this did not reach the level of statistical significance. On the basis of this analysis, we conclude that isoprinosine is safe. We further conclude that isoprinosine, as compared to placebo, appears to have no benefit if one analyses results in the entire patient population but there may be benefit in patients receiving the protocol medication for longer period of time and in patients with stage I disease. Banzet *et al.*[3] reported preliminary results of two randomized studies of isoprinosine. In the first study, 349 patients were randomized and there was no difference in the treated and control groups. In the preliminary report of the second study, 143 patients were evaluable, and there was a highly significant difference in the disease-free survival, but not in overall survival. Comparison of the final results of these studies of isoprinosine will be of interest.

1. Spitler LE. *J Clin Oncol* 1991; **9**: 736–740.
2. Quirt IC, *et al. J Clin Oncol* 1991; **9**: 729–735.
3. Banzet P, *et al. Melanoma Res* 1993; **3**: 293.

8

Adjuvant programme in malignant melanoma with hapten modified tumour cells, on patients with stage 3 disease who are rendered disease-free by resection of palpable large (> 3 cm) regional lymph node metastases

M. J. Mastrangelo and D. Berd

Division of Neoplastic Diseases, Department of Medicine, Jefferson Medical College, Philadelphia, PA, USA

Major impediments to successful tumour immunotherapy include weak immunogenicity of human tumour antigens and suboptimal methods of immunization. Our strategy to

overcome these impediments is multifaceted and includes BCG as an immunological adjuvant and pretreatment with low doses of cyclophosphamide to augment the development of cell-mediated immunity. Autologous tumour cells are conjugated with the hapten dinitrophenyl to generate helper T cells. We hypothesize that the T cell response to a strongly immunogenic, hapten-modified tumour antigen will be followed by development of immunity to unmodified tumour antigen. Use of this vaccine in patients with metastatic melanoma has produced one complete and four partial remissions among 46 patients (11%). Striking tumour inflammatory responses (marked erythema, warmth and tenderness of superficial metastases and the overlying skin) were commonly seen (about 50% of patients). Microscopic examination of inflamed tumours shows melanoma cells infiltrated with T lymphocytes the majority of which were CD8+. T cells derived from inflamed tumours exhibit a cell surface phenotype indicative of activation, ie HLA-DR+ and CD69+. Moreover, post-vaccine, inflamed tumour biopsies contain mRNA for γ interferon (5/8 specimens). Antitumour effects have been more apparent in patients with a smaller tumour burden. We have treated 78 patients with stage 3 melanoma, all of whom have been rendered disease-free by resection of palpable, large (>3 cm) regional lymph node metastases. Median follow-up is 22 months and 24 patients have been followed for 2 or more years. The 3-year disease-free survival and total survival of this group appear to be unusually high: about 55% and 62%, respectively. The published 5-year survival of patients treated with surgery alone is about 20%. Our own historical control group of 22 patients treated with unconjugated vaccine had 3-year disease-free survival and total survival rates of 28% and 30% respectively. In 7/10 patients who developed subcutaneous metastases as a first manifestation of recurrent melanoma, the histology of the tumours showed significant lymphocytic infiltration, sometimes associated with massive tumour necrosis. In summary, this is a seemingly active vaccine but has limited applicability to large patient populations because of the labour intensity of vaccine preparation, the unavailability of necessary quantities of autologous tumour and the plethora of regulatory issues.

9

Adjuvant therapy: from microbial immunostimulants to recombinant interferons

J. M. Kirkwood

University of Pittsburgh, PA, USA

The adjuvant therapy of melanoma has been pursued with a myriad of chemotherapeutic and biological agents. Chemotherapeutic approaches to date have failed to alter the relapse-free and overall survival of melanoma while a variety of leads from biological interventions have emerged, with recent early evidence of therapeutic benefit that may alter the standard of care for melanoma in the future. The analysis of studies of immunostimulants suggest that the activity of these agents was poorly understood, and trials inadequately constructed to test their role(s) in high-risk melanoma. The immunological prognostic variables that have been identified in high-risk melanoma during recent adjuvant trials of the agent OK432 have been defined, and may serve as the basis for more rational interventions with this class of agents in the future. The interferons (IFNs) are inducible glycoproteins produced in response to a variety of stimuli by most cells of the body. The three major types of IFN (α, β and γ) have been cloned and industrially produced for clinical investigations over the past decade. IFN α-2 has been most extensively evaluated for its potential role in the therapy of metastatic and resected high-risk melanoma. The antitumour activity of IFN α-2 in metastatic melanoma is well-documented, with response rates of 15–25% in single-institution trials. Adjuvant applications of IFN α-2 in high-risk T4 and N1 melanoma have been tested since 1984, and the first significant impact of therapy upon relapse-free survival in such patients was reported in the Eastern Co-operative Oncology Group (ECOG) trial EST 1684 in 1993.[1] At a median follow-up of 4.7 years, the continuous relapse-free survival of 38% of patients receiving IFN α-2 in the earlier ECOG trial EST 1684, as compared with 26% in patients who were observed in this trial, suggests the possibility of a curative effect of high-dose IFN α-2 in high-risk melanoma. The US Intergroup has further tested the same high-dose regimen for 1 year, in comparison to a less toxic low-dose regimen of IFN α-2 for 2 years, while the World Health Organisation has evaluated a similar low-dose regimen of IFN α-2 given for 3 years. These studies are ongoing and it is too early for meaningful assessment of survival impact.

1. Kirkwood J, Hunt M, Smith T, *et al.* A randomised controlled trial of high-dose IFN alfa-2b for high-risk melanoma: the ECOG trial EST-1684. *Proc Am Soc Clin Oncol* 1993; **12**: 390.

10

Efficacy of adjuvant interferon α2a therapy after radical surgery in melanoma patients with regional node metastases: the experience of WHO Melanoma Programme

F. Belli

On behalf of the WHO Melanoma Programme

From July 1990 to December 1993, 444 patients were accrued in a randomized multicentric clinical trial carried out by the WHO Melanoma Programme, with the participation of 23 centres, aimed at evaluating the efficacy of recombinant interferon-α 2a (rIFNα 2a) given at low dose (3 MU) three times a week for 3 years. Out of 444 patients 426 were evaluable: of these 208 were randomized to receive surgery only, and 218 surgery plus rIFNα 2a. The medium follow-up was 19 months. The 2-year disease-free survival was 46% (95% CI 36.4–54.8) in patients given interferon, but only 27% (95% CI 18.2–35.8) for those who received surgery alone ($P=0.01$, log-rank test). Multivariate analysis (Cox's regression model) of factors that influenced disease-free survival indicated that in addition to adjuvant interferon therapy, the number of positive nodes ($P=0.0003$) and sex ($P=0.03$) were independent determinants of disease-free survival. Analysis of disease-free survival in males and females by age indicated a complex relationship between age, sex and IFNα-2a therapy. Improved survival in males with increasing age given IFN was seen. In females the opposite trend was seen,

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Vaccine Trials for the Clinician: Prospects for Tumor Antigens

SUSANNE OSANTO

Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands

Key Words. *Vaccines · Vaccination · Tumor antigens · Cytotoxic T cell lymphocytes · Peptides · T lymphocyte epitopes · Histocompatibility antigens Class I · Antigen-presenting cells · Gene therapy · Genetic engineering*

ABSTRACT

Recent insights in antigen presentation, the identification of human tumor antigens, and the demonstration of MHC class-I-restricted cytotoxic T lymphocyte (CTL) recognition of peptides encoded by tumor antigen have renewed the interest and enthusiasm for the development of cancer vaccines. Melanoma serves as a paradigm of an immunogenic human tumor, and several tumor antigens, including MAGE, MART-1/Melan-A and gp100, recognized by CTLs, have now been isolated. Candidate antigens for novel vaccine trials may include HLA class-I-binding tumor peptides that serve as CTL epitopes, whole tumor protein, or DNA-based vaccines. Requirements for the use of peptides are that the patient's tumor presents the relevant CTL epitopes as used in the vaccine and expresses the appropriate MHC class-I-restricting molecule. Immunological monitoring may be facilitated when using peptide-based vaccines. Because optimal presentation of tumor antigens may depend on provision of appropriate costimulatory signals, it may be more advantageous to administer professional antigen-presenting cells (APCs), such as dendritic cells (DCs) pulsed with tumor peptide or protein, to cancer patients.

Developments in molecular genetics have led to a new approach in vaccines consisting of cancer cells genetically engineered to express immunomodulatory molecules. This may result in increased antitumor responses to both gene-modified as well as unmodified tumor cells.

The therapeutic approach is extended to vaccination trials with recombinant viruses containing the genes encoding tumor antigens, minigenes containing multiple CTL epitopes, or double recombinant vectors engineered to express both the tumor antigen and immunostimulatory molecules.

Clinical peptide, protein, and DNA-based vaccine trials have recently been initiated. Thus far, exciting clinical remissions were obtained in melanoma patients following vaccination with HLA-A1-binding MAGE-3 peptide and in B-cell lymphoma patients immunized with autologous DCs pulsed with anti-idiotype protein, i.e., the individual patient's unique tumor antigen. Also, following injection of foreign HLA-B7 DNA into cutaneous melanoma metastases, T-cell migration into treated lesions and enhanced cellular immunity at the site of the tumor were shown in some patients. These encouraging results suggest that effective new vaccines in cancer will be identified. *The Oncologist 1997;2:284-299*

INTRODUCTION

With the maturing of our insights into the biology of cancer and basic immunological mechanisms, we now have opportunities to rationally develop vaccine approaches against cancer. The idea of developing a cancer vaccine has been a dream of immunologists for years and is based on the concept that tumors possess distinct antigens that should be recognized by the immune system.

Different mechanisms may be used to amplify tumor-specific immune responses. In the absence of well-defined tumor regression antigens, attempts to develop cancer vaccines have made use of live or irradiated allogeneic or autologous tumor cells or tumor cell lysates, either alone or mixed with adjuvants such as *bacillus Calmette-Guérin* (BCG) and *Corynebacterium parvum* [1-13]. These vaccination studies have been largely unsuccessful, although in some reports, the remissions associated with disease-free survival were shown to correlate with the return of delayed-type hypersensitivity (DTH) responses to recall antigens and the development of a DTH response to autologous tumor cells. Also, well-defined immunogenic molecules such as gangliosides (glycosphingolipids anchored in the lipid bilayer of plasma membranes and overexpressed in melanomas and neuroblastomas) that are derived from melanoma cells or other sources [14-17] and anti-idiotypic

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antibodies carrying the "mirror image" of the antigen [18-20] have been administered to cancer patients as vaccine.

More recently, vaccination trials have been initiated using tumor cells that were genetically modified by DNA sequences encoding a variety of immunomodulatory molecules to increase their immunogenicity. Unraveling of the cellular basis of antigen recognition has fueled the current interest in exploration of tumor peptides for vaccination against cancer, particularly in melanoma. Rational vaccine protocols may include tumor peptide or (protein) antigen, administered alone or pulsed onto dendritic cells (DCs). The following overview will present the background and current state of immunobiology-driven vaccine development for cancer aiming at T cell-mediated antitumor responses.

Antigen Recognition by T Cells

The immune system has over time generated two arms to defend the body: the humoral and the cellular immune response. T lymphocytes play a central role in the cellular immune response. Antibodies recognize antigens as native, folded protein at the cell surface, whereas T cells recognize antigen as a fragment of protein (peptide) complexed with a major histocompatibility complex (MHC) molecule on the surface of cells (Fig. 1) [21, 22]. The MHC molecules are highly polymorphic, and the different alleles have distinct peptide-binding specificity. Sequencing of peptides eluted from MHC molecules resulted in the discovery of allele-specific motifs which correspond to critical anchor residues; these residues fit into specific pockets of MHC molecules [23, 24].

T lymphocytes play a central role in the cellular immune response.

Intracellular proteins in the cytosol are cleaved by proteosomes into short peptides comprising 8 to 10 amino acids. These peptides are transported via the specialized transporter associated with antigen processing (TAP) into the endoplasmic reticulum where they bind to newly synthesized MHC class I molecules. After binding, the complex is transported through the Golgi apparatus to the cell surface, where it can be recognized by cytotoxic T cells ($CD8^+$) (Fig. 1). Since all endogenous intracellular proteins can be presented to the immune system in this way, any tumor-specific structure may function as a potential tumor-specific antigen and be recognized by T cells.

Antigen-presenting cells (APCs) express high levels of MHC class I and II and accessory/costimulatory molecules [25, 26], and migrate to central lymphoid organs where optimal priming of T cells can occur and immune responses are initiated. DCs are the most potent APCs in the body that play a major role in initiating immune responses such as activation of MHC-restricted T cell responses and the formation of T cell-dependent antibodies. APCs take up extracellular proteins by either endocytosis or phagocytosis. Foreign proteins are degraded into peptides in acidified endosomes, where they bind to newly synthesized MHC class II molecules, which are specifically targeted to this compartment. The MHC-peptide complex is then brought to the cell surface where it can be recognized by $CD4^+$ helper T cells. Depending on the helper T cell that binds to the complex, B cells are stimulated and antibody production augmented. Like most cells, APCs use their MHC class I molecules to present peptides from endogenous proteins. Evidence has now emerged that in addition to presenting endogenous peptides, a subset of APCs can also acquire and present exogenous proteins on MHC class I molecules [27-31]. The discovery of this second antigen-presenting pathway provides an opportunity to develop new kinds of protein-based vaccines that will result in initiation of cytotoxic T lymphocyte (CTL) responses to class-I-binding peptides derived from exogenously administered proteins. While intact proteins need to be processed to generate antigenic peptides, soluble peptides can bind directly to a small fraction of empty class I or class II molecules present on the cell surface.

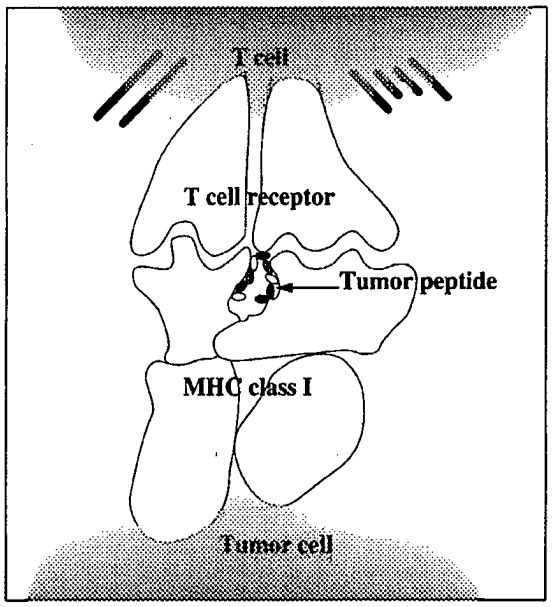

Figure 1. Scheme for the recognition of a tumor peptide complexed to MHC class I molecule by a CTL.

Human Tumor Antigens Recognized by T Cells

Melanoma is the most striking example of a non-virus-induced immunogenic tumor in man that is able to elicit T cell-mediated antitumor immunity in vivo. The majority of human tumor antigens defined by T cells have been identified utilizing patients' T lymphocytes as effector cells and tumor cells obtained from autologous (metastatic) tumor deposits as targets. Several investigators have isolated cross-reactive tumor-specific CTLs from peripheral blood, lymphocytes, or tumor-infiltrating lymphocytes of melanoma patients, and these CTLs are able to recognize common tumor antigen expressed in melanomas that share the restricting HLA class I allele [32-35]. Since 1991, a number of genes encoding human melanoma antigens recognized by T cells have been cloned utilizing melanoma-reactive CTLs [36-44].

Three types of T cell-defined antigens encoding for several HLA class-I- or class-II-binding peptides have now been identified in melanoma (Table 1) and these antigens are targets for vaccination (Table 2). The first group of antigens is expressed in melanoma but also in other cancers (e.g., melanoma antigen [MAGE], BAGE, GAGE). The second group of antigens is specific for melanocytic differentiation, and these antigens are shared by melanoma and melanocytes (e.g., tyrosinase, MART-1/Melan-A, and gp100). The third group of antigens is unique, resulting from point mutations expressed by the individual patient's tumor (e.g., MUM-1, CDK4).

The first melanoma antigen that was identified is MAGE-1. MAGE-1, -2, and -3 were the original family of human melanoma-specific antigens that were molecularly identified using a DNA library to clone the gene [36, 43]. MAGE-1 antigen is restricted by HLA-A1 or Cw16 [45, 46], while MAGE-3 yields peptides recognized by HLA-A1- or HLA-A2-restricted CTLs [47, 48] (Table 1). The MAGE genes are not expressed in normal adult tissues except testis, but are also expressed in carcinomas of breast and lung carcinomas [49] (Table 2). The function of MAGE has not been elucidated yet. More recently, other genes called BAGE [37] and GAGE-1 and -2 [38] were identified in melanoma and shown to be expressed in other cancers, but not in adult tissue except the testis. BAGE and GAGE were shown to be restricted by HLA-Cw16 and HLA-Cw6, respectively (Table 1).

The melanoma antigens MART-1/Melan-A, gp100, tyrosinase, and tyrosinase-related protein (TRP1 or gp75) represent differentiation antigens expressed by normal melanocytes. Tyrosinase is a key enzyme in the melanin synthesis pathway in pigmented cells; the functions of the other genes are not known. MART-1/Melan-A, gp100, and tyrosinase each yield several HLA-A2-binding CTL epitopes, but the four differentiation antigens are also recognized by HLA-A24, -A31, and -B44-restricted CTLs [40-42, 44, 50-60] (Table 1). HLA-A2 is the most frequent MHC class I allele in

Table 1. Human melanoma tumor antigens detected by T lymphocytes

Tumor antigens	MHC-restriction	Peptide sequence	References
Melanoma antigens			
Melanoma differentiation antigens shared in melanoma and melanocytes			
MART-1/Melan-A	HLA-A2	AAGIGILTV	[50]
	HLA-A2	ILTVILGVL	[54]
gp100	HLA-A2	ITDQVPFSV	[51]
	HLA-A2	VLYRYGSFSV	[51]
	HLA-A2	KTWGQYWQV	[51, 52]
	HLA-A2	LLDGTATLRL	[51, 53]
	HLA-A2	YLEPGPVTA	[51, 64]
Tyrosinase	HLA-A2	MLLAVLYCL	[56]
	HLA-A2	YMNGTMSQV	[56]
	HLA-A24	AFLPWHRLF	[57]
	HLA-B44	SEIWRDIDF	[58]
	HLA-DR4	QNILLSNAPLGPQFP	[62]
	HLA-DR4	SYLQDSDPDSFQD	[62]
TRP-1 (gp 75)	HLA-A31	MSLQRQFLR	[59]
Tumor-specific antigens also expressed in other cancers			
MAGE-1	HLA-A1	EADPTGHSY	[45]
	HLA-Cw16	SAYGEPRKL	[46]
MAGE-3	HLA-A1	EVDPIGHLY	[47]
	HLA-A2	FLWGPRALV	[48]
BAGE	HLA-Cw16	AARAVFLAL	[37]
GAGE-1, -2	HLA-Cw6	YRPRPRRY	[38]
Tumor-specific, mutated gene products			
MUM-1	HLA-B44	EEKLIVVLF*	[66]
CDK4	HLA-A2	ACDPHSGHFV*	[67]
β-catenin	HLA-A24	SYLDSGIHF*	[68]
Antigens in epithelial cancers			
HER-2/neu	HLA-A2	KIFGSLAFL	[79]
HER-2/neu	HLA-A2	IISAVVGILL	[80]
CEA	HLA-A2	YLSGANLNL	[102]
HPV-E6, -E7	HLA-A2	YMLDLQPETT	[88]

* Mutations are underlined.

Caucasians and appears to be the predominant restriction element for an antimelanoma-directed immune response. MART-1/Melan-A, and gp100 appear to be recognized by a high percentage of tumor-infiltrating lymphocytes (TILs) and tumor-reactive CTL lines obtained from HLA-A2 patients [40, 41, 50-53]. Tyrosinase has also been identified as recognized by $CD4^+$ T cells in a class II-restricted manner [61, 62] (Table 1). Some of the peptides derived from the differentiation antigens were isolated by elution of peptides from the MHC molecules at the cell surface of melanoma cells [63, 64]. The immunogenicity of these melanocyte differentiation antigens demonstrates that an immune mechanism against nonmutated self-antigens with limited tissue distribution can be mounted in cancer patients and that it is indeed possible to alter the state of tolerance to self-antigens [65].

The third group of antigens comprises mutated gene products, namely mutated MUM-1, CDK4, and β-catenin, recognized by autologous lymphocytes from individual patients [65-68] (Table 1). A CTL-epitope encoded by a mutated intron was recently identified and the gene product resulting from incomplete mRNA splicing was named MUM-1. Mutated cyclin-dependent kinase 4 (CDK4, a protein involved in cell cycling) encodes for another CTL epitope. β-catenin is involved in cell-cell adhesion.

T-cell mediated antitumor reactivity has also been found in other types of malignancies including breast carcinoma, ovarian and renal cell carcinoma [69-83]. Targets for vaccination include viral products in virus-induced malignancies, fusion proteins derived from chromosomal breakpoints, and the products of oncogenes that are either mutated or overexpressed in malignant tumors [84-101] (Table 2).

Human papilloma virus (HPV) type 16 (HPV16) is strongly associated with cervical carcinogenesis. The HPV16 E6 and E7 oncoproteins are constitutively expressed in the majority of cervical tumor cells and are, therefore, attractive targets for CTL-mediated immunotherapy. CTL reactivity against HPV proteins has also been found in cervical cancer patients. Although a number of HLA-A2-binding peptides encoded by the viral oncogenes E6 and E7 from HPV type 16 are identified [84, 86, 87], only a small number of patients with HPV16-associated cervical lesions were shown to have a natural CTL response to these peptides [88]. This suggests that in many cervical cancer patients a CTL response against such proteins can be induced and that HPV16 E6 or E7 peptide-based vaccination strategies may be useful.

The predominance of oncogene activation in human cancer makes the mutated oncogene products attractive candidates for immunotherapy [89, 90]. Somatic point mutations in ras oncogenes are frequently found in pancreatic (90%) and other gastro-intestinal adenocarcinomas. Mutant ras peptides are therefore a candidate vaccine for specific immunotherapy in pancreatic and colon carcinoma patients [90]. Both $CD4^+$ and $CD8^+$ T cell clones recognizing mutant ras have been identified in patients with colorectal cancer [91-93]. Furthermore, human HLA class-I-restricted CTL responses against mutant ras could be induced in vitro [94]. However, the CTLs generated in vitro did not show lytic activity against tumor cells expressing mutant ras. The p53 protein represents another potential target as p53 mutations, leading to enhanced expression of p53, are common in several cancers, e.g., colon, lung, and breast carcinoma [95-97].

Chromosomal translocations may result in the generation of fusion genes such as the *bcr-abl* gene in chronic myelogenous leukemia. CTLs specific for bcr-abl peptides that bind to HLA class I molecules can be generated [98, 100, 101]. Fusion proteins are common in human malignancy and are candidate antigens for vaccine trials.

CTL reactivity against HER-2/neu has been found in patients [79, 80]. HER-2/neu is a growth factor receptor homologous to an epidermal growth factor receptor that is overexpressed in about one-third of breast carcinomas and also in ovarian, lung, and colon adenocarcinomas. In breast cancer, HER-2/neu overexpression has been correlated with

Table 2. Human tumor antigens that are potential targets for vaccination in cancer patients

Type of antigen	Type of cancer
Tumor-specific antigens	
MAGE-1, -2, -3	Melanoma, breast, lung cancer
BAGE	Melanoma, breast cancer
GAGE-1, -2	Melanoma, breast, lung, bladder cancer
Idiotype antibody	B-cell malignancy
Mucin-1	Breast, ovarian, pancreatic cancer
Differentiation antigens	
Tyrosinase	Melanoma
TRP-1	Melanoma
MART-1/Melan-A	Melanoma
gp100	Melanoma
Prostate-specific antigen	Prostate cancer
Mutated oncogenic or fusion protein	
ras	Gastrointestinal, lung cancer
p53	Colorectal, breast, lung cancer
bcr-abl	Chronic myelogenous leukemia
Overexpressed proteins	
HER-2/neu	Breast, ovarian, lung cancer
Viral proteins	
HPV E6, E7	Cervical cancer
Epstein-Barr virus	Burkitt's lymphoma, nasopharyngeal cancer

HPV = human papilloma virus

poorer prognosis. Also, carcinoembryonic antigen (CEA) [102] may potentially be a good candidate for vaccination in patients with colorectal cancer. In patients with overexpression of HER-2/neu, both T cells and antibodies reactive to HER-2/neu could be demonstrated [103]. Animal studies indicate that vaccines consisting of subdominant epitopes derived from these self-proteins may elicit an effective immune response [104].

MUC-1 (Mucin 1, PEM) is a large, molecular, tumor-specific carbohydrate antigen on the cell surface that is aberrantly glycosylated and upregulated in breast, ovarian, colon, and pancreatic carcinoma. MHC-unrestricted MUC-1 specific CTLs have been isolated from breast, ovarian, and pancreatic cancer patients [69, 71].

The identification of common tumor antigens implies that we do not depend solely on the use of autologous tumor cells as a vaccine in cancer patients. It justifies the use of both autologous as well as allogeneic HLA-matched tumor cells and tumor antigens as vaccines. Such vaccines may be applicable in a considerable number of cancer patients.

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Gene-Modified Tumor Cell Vaccines

In the early 1990s, various investigators reported enhanced immunogenicity of tumor cells following genetic modification with cytokine cDNA. In several models, interleukin 2 (IL-2) gene-modified tumor cells as well as the wild-type, unmodified parental tumor cells were rejected [105-108]. Similar results have been obtained following the use of tumor cells engineered to secrete other cytokines, e.g., IL-4, IL-7, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), G-CSF, and GM-CSF [109-116]. Most reports indicate that this approach is highly effective in protecting animals from subsequent tumorigenic doses of non-modified tumor cells, but few animal studies indicate that this approach is also effective in eradication of already-established tumors. Similar strong antitumor responses were obtained following vaccination with tumor cells engineered to express foreign MHC genes, class I or II, or the B7 (costimulatory molecule) cDNA [117-125].

The effector cells that mediate the observed reduced tumor regression may differ depending on the model studied. Since the first human gene marking was approved and conducted in the U.S. [126], several gene therapy protocols have been initiated there and in Europe. The advantage of a genetically modified autologous cell vaccine is that it contains the whole collection of tumor proteins and therefore has the greatest chance of inducing an immune response against relevant tumor antigens. However, growing autologous tumor cells in vitro to establish tumor cell lines is time-consuming and often unsuccessful. Following administration, allogeneic tumor cells that share an HLA class I allele may in vivo either directly present shared immunodominant tumor peptides to class-I-restricted CTLs or first be degraded and processed by professional APCs (Fig. 2A and Fig. 2B). These APCs will process and select the appropriate epitopes, which will enter the class II or even the class I route to stimulate the patient's $CD4^+$ or $CD8^+$ T cells.

We have initiated a clinical study in metastatic melanoma patients to evaluate the toxicity and antitumor efficacy of weekly s.c. injections of IL-2-secreting, allogeneic melanoma cells that share one or more HLA class I alleles with the patient [127]. We have observed inflammatory reactions and regression of distant metastases, and, in some cases, significant increases in antimelanoma CTLs frequencies. There are now several ongoing human gene therapy trials employing a similar approach with tumor cells engineered to express costimulatory molecules or secrete cytokines for the treatment of patients with cancer [128, 129]. In one study, a limited antitumor T cell response was found following vaccination [130]. Now that the feasibility of these strategies has been demonstrated, they should be applied in the adjuvant setting in high-risk patients because the approach of gene-modified tumor cell vaccines will only be successful when minimal tumor burden exists.

Peptide-Based Cancer Vaccines

The advantages of synthetic peptides are that the preparations show chemical consistency from batch to batch and that immunological monitoring of defined T cell epitope is easier. Another advantage is the relatively simple and inexpensive production of large quantities and the possibilities of constructing multi-epitope vaccines by combining CTL epitopes derived from different tumor antigens.

A disadvantage of peptide vaccines is the restriction of each peptide to one HLA molecule [131]. For a peptide-based vaccine to be widely applicable, it will be necessary to identify multiple peptide epitopes that are presented by all the major MHC class alleles. Persistence of peptide antigen

in vivo will be limited by clearance and degradation. The presence of serum peptidases may alter the antigenicity of peptides or rapidly inactivate peptides. To raise the immunogenicity, peptides can be injected with adjuvants, in liposomes, or by direct attachment of lipids [132]. Immunization with DCs, the most powerful professional APCs that are able to prime naive CTLs in vitro and in vivo, may represent a cancer vaccine that is superior over a vaccine containing peptide alone [133-136].

Peptide-based vaccine therapies in melanoma patients have recently been initiated in a few centers in Europe and the U.S. *Marchand et al.* [137] reported significant tumor regressions (including one complete remission) in 3 out of 12 HLA-A1-positive tumor-bearing melanoma patients who were immunized with s.c. injections of the synthetic HLA-A1-binding MAGE-3 peptide. These results are remarkable, as the peptide was injected without adjuvant, and tumor responses in the absence of adjuvant had not been anticipated. *Jaeger et al.* [138] vaccinated six HLA-A* metastatic melanoma patients intradermally with multiple CTL epitopes, i.e., peptides derived from MART-1/Melan-A, tyrosinase, and gp100/Pmel 17. In addition, the influenza matrix peptide was administered as a control. DTH reactions were observed in five out of six patients. Generation of peptide-specific CTLs was documented against MART-1/Melan-A-derived peptide epitopes, the tyrosinase signal peptide, and the influenza matrix peptide after vaccination. No tumor regressions were observed.

A number of clinical studies under the guidance of *Rosenberg* employing synthetic HLA-A2-binding peptides derived from the melanoma differentiation antigens MART-1/Melan-A and gp100 are in progress. In a phase I study, 28 melanoma patients were immunized with escalating doses of the immunodominant gp100 nonapeptides $gp100_{aa154-162}$, $gp_{aa209-217}$, and $gp100_{aa280-289}$, administered s.c. in incomplete Freund's adjuvant [139]. Administration of more than two immunizations with the $gp100_{aa209-217}$ and $gp100_{aa280-289}$ peptides appeared to further enhance the patient's immune reactivity against the 209 and 280 epitopes. No 154 peptide-specific activity could be demonstrated in the patients immunized with $gp100_{aa154-162}$. Because the immunodominant gp100 peptides have relatively low binding affinity to HLA-A2, peptides modified at the HLA-A2-binding anchor positions, but not at T cell receptor (TCR) contact residues, were selected based on MHC binding affinity [140]. Two of these peptides, one containing an amino acid substitution at position 2 of $gp100_{aa209-217}$, and one containing an amino acid substitution at position 9 of $gp100_{aa280-289}$, are high-affinity binding peptides and seem to be more immunogenic than the native epitopes. A clinical study with these modified high-affinity binding peptides is now in progress.

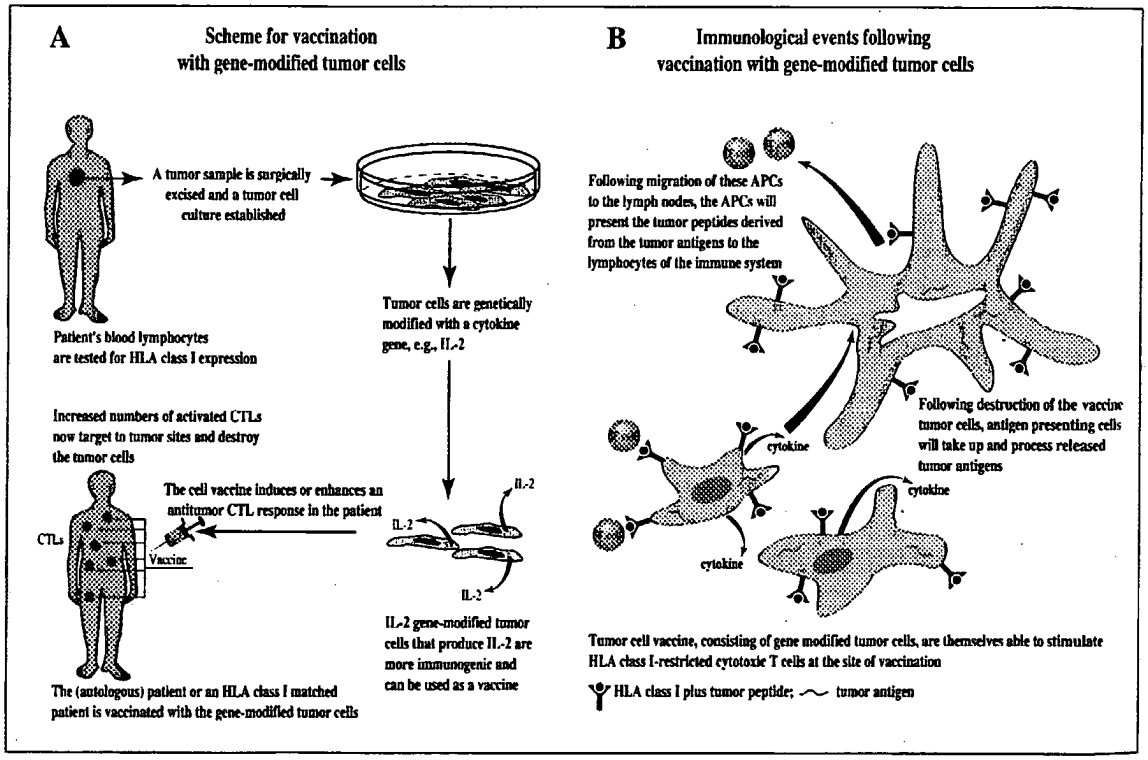

Figure 2.

In another study, three patients with advanced metastatic melanoma were vaccinated with autologous cultured DCs pulsed with an HLA-A1-binding MAGE-1 nonapeptide. MAGE-1 peptide-specific CTLs were demonstrable after, but not prior to, vaccination, and these CTLs were capable of lysing HLA-A1 MAGE-1-positive melanoma cells in vitro [141]. No major therapeutic responses were noted, possibly because of the advanced stage of the disease.

Others investigated the potential of vaccinating cancer patients with peptides derived from mutant ras and showed that vaccination of end-stage pancreatic carcinoma patients with mutant ras peptide-pulsed APCs from peripheral blood resulted in a transient ras-specific proliferative T cell response in some of these patients [142, 143].

Protein Vaccines

Several lines of evidence may support the delivery of entire proteins rather than the use of a peptide-based vaccine. Use of a peptide vaccine is limited to single epitopes. The use of whole protein vaccines may be advantageous over peptide vaccines in that it provides a wider range of multiple MHC class I (and class II) epitopes, several T cell epitopes binding a single MHC allele, and also several T cell epitopes binding to different MHC class I alleles. Therefore, whole proteins may provide T cell epitopes that have not been identified in the context of other common HLA alleles.

B cell malignancies are unique in that they express abundant tumor-specific cell-surface antigen (immunoglobulin) which is not shed from the neoplastic cells. As these malignancies are monoclonal, all the cells of a given tumor express identical immunoglobulin receptors, making them a suitable tumor-specific target for immunotherapy. However, each lymphoma has a unique idiotypic immunoglobulin, and anti-idiotypic strategies must therefore be tailored to individual patients.

A clinical trial was initiated to evaluate the efficacy of tumor-specific idiotype protein-pulsed autologous DCs in the treatment of B cell lymphoma [144]. Tumor biopsies were obtained from patients with B cell lymphoma, and the immunoglobulin (idiotype protein) produced by each tumor was obtained by cell fusion techniques. DCs were isolated from the peripheral blood of patients with lymphoma by leukapheresis and density-gradient centrifugation. Four patients with follicular B cell lymphoma received a series of three or four infusions of antigen-pulsed DCs followed by s.c. injections of soluble antigen two weeks later. All patients developed measurable antitumor cellular immune responses. In addition, three of the four patients experienced clinical remissions of the disease (two complete remissions and one partial remission). This study has demonstrated the ability of antigen-pulsed DCs to stimulate clinically relevant immune responses in humans.

Recombinant Viral Vectors

In contrast with tumor cells that are often poorly immunogenic, viruses or viral extracts can elicit a strong specific and lifelong immunity. In animal models, it has been shown that model tumor antigens presented by viruses are highly immunogenic, whereas the same model antigen presented by tumor cells is not [145]. Several viruses, including recombinant vaccinia virus, fowlpox virus, and adenovirus encoding model tumor antigens, have been shown to express antigens within the cytoplasm of infected cells, resulting in the induction of murine immunity. However, immunization with live attenuated or recombinant viruses may also pose safety problems due to their infectious nature. Furthermore, the delivery of whole genes encoding tumor antigens that are involved in carcinogenesis may lead to malignant transformation of recombinant-virus-infected cells. For instance, viral vector vaccines containing the functional HPV E6 and E7 oncogenes, mutated oncogenes, aberrant fusion proteins, or tumor antigens (such as MAGE, GAGE, and BAGE, whose functions are still unknown), should be considered unsafe. By introducing only the CTL epitopes derived from such tumor antigens into viral vectors, T cell immunity may be induced without introducing potential hazards. Nonviral delivery systems are also being developed as an alternative to current delivery strategies employing viral-mediated gene therapy approaches.

Recombinant viral vectors can now be constructed in such a way that the presentation of the antigens to the T cells is optimized. For instance, recombinant viruses were constructed containing minigenes encoding antigenic peptides with an amino-terminal endoplasmic reticulum insertion sequence (circumventing requirements for proteolysis and transport) and were shown to greatly enhance the $CD8^+$ CTL immune response [146-148]. Furthermore, vaccinia viruses carrying string-of-beads constructs containing the genetic code for multiple CTL epitopes are constructed, and each epitope within the polyepitope protein has been shown to be processed and presented for CTL-mediated lysis. Such polyepitope CTL vaccines have been shown to be effective in eliciting the desired immune response [149-151]. In addition, molecular technology now enables us to construct viral vectors that allow specific targeting of an antigen to the endosomal and lysosomal compartments, resulting in enhanced presentation via the MHC class II pathway and subsequent recognition of the target antigen by $CD4^+$ T cells [152, 153]. Moreover, viral vectors can be constructed which encode not only the tumor antigen of interest but also for cytokines or other costimulatory molecules that can facilitate the activation of a powerful cellular immune response [154-157].

In one of the first clinical vaccination studies against a (self) tumor antigen employing a vaccinia viral construct containing the carcinoembryonic antigen, *Tsang et al.* [102] were able to show that tolerance can be broken by vaccination. CTL responses to a specific CEA epitope were induced and the CTLs were able to lyse tumor cells expressing CEA. In this study, in spite of the enhanced immunoreactivity, no clinical benefit was obtained, possibly because of the advanced stage of the disease in the patients. A trial using the same recombinant vaccinia-CEA vector is now performed in gastrointestinal cancer patients with minimal disease.

Recently, *Borysiewicz et al.* [158] reported a first clinical trial in late-stage cervical cancer patients in which patients were immunized by dermal scarification with a recombinant vaccinia viral construct encoding modified HPV 16 and 18 E6 and E7 protein sequences. All patients mounted an antivaccinia antibody response. Three of eight patients developed an HPV-specific antibody response. HPV-specific cytotoxic T lymphocytes were detected in one of three evaluable patients.

DNA Vaccination

DNA delivering methods have been shown in animals to be efficient enough to raise immune responses [159-164]. With naked DNA vaccines, the host cell manufactures the protein and CTL epitope. Plasmids are easy to manipulate and can accommodate large sequences of foreign DNA. They can be produced at a high level of purity and are associated with low immunogenicity. DNA-based vaccinations with naked DNA encoding tumor antigen may supersede the more complex technology of other gene therapy protocols.

DNA-based vaccinations with naked DNA encoding tumor antigen may supersede the more complex technology of other gene therapy protocols.

The gene gun gene delivery system [165-167] is an effective means of introducing antigen-encoding expression vectors into the epidermis. The immunization of the skin results in temporal presence of DNA and expression of antigen, but elicits humoral and cellular immune responses and protective immunity. The skin is rich in DCs and ballistic cutaneous genetic immunization may result in in vivo transfection of skin-derived DCs. Endogenously synthesized antigen can access the MHC class-I-restricted pathway of transfected DCs. Following migration to regional lymphoid organs, the DCs can present the tumor antigen to T cells with appropriate costimulatory signals for T cell activation.

DNA immunization can produce long-term humoral and cellular immune responses qualitatively similar to that of live attenuated vaccines [161-164]. Priming of MHC class-I-restricted $CD8^+$ T cells may require ingestion, processing, and presentation of peptides derived from the expressed protein on MHC class I molecules by host APCs (cross-priming) in vivo [168-170].

Although treatment of B cell lymphoma patients with anti-idiotype antibody has been demonstrated to be successful [171], analysis of lymphoma cells at the time of relapse pointed to somatic mutation in the idiotypic V genes (idiotypic escape) as an important mechanism by which the neoplastic clones survive exposure to an anti-idiotypic monoclonal antibody. Importantly, loss of surface immunoglobulin does not appear to occur. A phase I trial has been initiated to test the safety of the genetic approach to personalized idiotypic vaccination with DNA encoding the idiotypic V gene [172]. Variable region gene sequences coding for the lymphoma idiotype were isolated directly from biopsy material by polymerase chain reaction (PCR) amplification, cloning, and DNA sequence analysis of the cloned PCR product.

Another approach is to enhance the antitumor immune response by in vivo injection of a foreign HLA class I gene, i.e., HLA B7, by DNA liposome complexes directly into tumor deposits. *Nabel* and coworkers showed that the introduction of HLA-B7 DNA into cutaneous melanoma metastases by direct injection brought about a 10%-15% transduction of the tumor cells [173]. The HLA-B7 protein expression could be demonstrated to be expressed in the tumor cells near the site of injection. No systemic toxicity was observed, and regression of a distant lung metastasis in one of the five reported patients suggests that allogeneic effects may indeed enhance antitumor immune response. In a more recent update on 10 patients, *Nabel et al.* [174] reported that in most patients HLA-B7 gene transfer did not markedly alter the frequency of circulating tumor-specific CTL in peripheral blood, whereas T cell migration into treated lesions was enhanced in the majority of patients, and tumor-infiltrating frequency of lymphocyte reactivity was enhanced in the two patients studied. In one patient, subsequent treatment with tumor-infiltrating lymphocytes derived from gene-modified tumor resulted in a complete regression of the residual disease. These data suggest that immunological monitoring of lymphocytes from the peripheral blood compartment does not provide accurate information and, instead, T cells from metastatic sites should be followed.

Guidelines to Clinical Applications of Cancer Vaccines

Melanoma serves as a paradigm for tumor immunology, and cancer vaccine trials will initially focus on melanoma. Various CTL epitopes and the encoding tumor antigens have been identified in melanoma, enabling the clinician to explore several strategies, as outlined in Table 3. Clinical trials will have to demonstrate whether a single strategy will prove to be superior. In animal studies, immunotherapeutic approaches are more likely to be successful when the tumor load is relatively small. Based on this fact, it may be important to select patients with small tumor burden, for instance, high-risk patients following excision of a thick primary melanoma or following regional lymph node dissection (AJCC stage IIB and III melanoma patients). The disadvantage of such adjuvant studies may be the long period of time and large numbers of patients required to demonstrate the efficacy of the approach. For this reason, ability to monitor effective immune responses becomes even more critical. It may be useful to determine before entry to the study whether the patient's cellular immunity is able to react to known antigens by performing a simple delayed hypersensitivity test to recall antigens.

In order to enable an effective T cell-mediated antitumor response to be mounted in the patient, tumor cell surface expression of MHC class I molecules that are able to present tumor peptides is a prerequisite. Thus, MHC class I expression of patients' tumor cells has to be determined (Table 4).

The choice of tumor antigen to be administered as a vaccine, whether formulated as peptide, protein, gene, or minigene, has to be based on the tumor antigen expression in the patient's tumor cells. The frequent expression in melanoma of differentiation antigens and shared tumor antigens enables the use of tumor antigen-based vaccines. The infrequently expressed unique antigens that arise from point mutations provide candidate vaccines for individual patients. However, they require identification in individual patients and are therefore less attractive candidates for clinical trials.

All melanoma patients are candidates for vaccination with either gene-modified, whole-tumor cell vaccines, tumor protein vaccines, or vaccines consisting of DNA encoding the tumor antigen. In HLA-typed HLA-A2 or HLA-A1 melanoma patients, various peptide-based strategies are possible (Tables 1 and 4). The existence of multiple HLA-A2-binding epitopes in gp100, MART-1/Melan-A, and tyrosinase, and of HLA-A2-binding peptide derived from MAGE-3, enables the use of multivalent peptide vaccines in HLA-A2-positive melanoma patients. HLA-A1 positive patients are eligible for MAGE-1 and MAGE-3 binding peptide-based vaccines.

Until now, only a few CTL epitopes (mainly HLA-A2-binding peptides) derived from various tumor antigens have been identified. Because HLA-A2 molecules are expressed in 40% to 50% of Caucasians, HLA-A2-binding peptides may have therapeutic potential in a

Table 3. Cancer vaccines aimed at induction or enhancement of a T cell-mediated antitumor response

Whole tumor cell vaccines

- Unmodified
- Gene-modified with genes encoding cytokines, costimulatory molecules or other genes to enhance the immune response

Peptides (single or multivalent peptide vaccine)

- Alone
- With adjuvants
- Linked to lipids, liposomes
- Pulsed onto APCs

Proteins

- Alone
- With adjuvants
- Linked to lipids
- Pulsed onto APCs

DNA-encoding tumor antigens

- Gene gun for intradermal injection
- Intramuscular injection
- Linked to lipids
- APCs transduced with DNA-encoding tumor antigens or a minigene containing multiple CTL epitopes
- Recombinant viruses encoding tumor antigens
- Recombinant viruses encoding tumor antigens plus genes encoding cytokines, costimulatory molecules, or other genes to enhance the immune response

Table 4. Monitoring of vaccine trials in cancer patients

Prestudy screening

- Stage of the disease and performance status
- DTH test to recall antigens
- Establish tumor phenotype
  - MHC class I and II
  - Tumor antigen expression (RT-PCR)
- HLA typing (HLA-A2 or -A1 for known HLA-A2 and -A1-binding peptides)
- Obtain peripheral blood lymphocytes (buffy coat or leukapheresis)

Monitoring immunological response

- DTH testing of peptide(s) or tumor cells used
- Antitumor or peptide-specific CTL precursor frequency analysis in peripheral blood lymphocytes or TILs
- Determination of CTL responses in bulk cultures or at the clonal level in peripheral blood lymphocytes or TILs
- TCR expression in peripheral blood lymphocytes or TILs

significant number of cancer patients. However, the arsenal of peptides may be extended to other new epitopes. Based on predicted HLA class-I-binding motifs that were first described for HLA-A2 and now for HLA-A1, -A3, -A11, -A24, and -B7, as well as other alleles [23, 24, 131, 175], we are now able to predict which peptides derived from a protein with known amino acid sequence will bind to certain class I alleles. It is thus possible to have such predicted peptides synthetically synthesized and to test for binding to the appropriate class I molecule. Such peptides could then be tested for immunogenicity in vitro or in HLA transgenic animal models, and also used in vaccine programs. The identification of a set of tumor-specific peptides that can bind to the previously mentioned HLA alleles enables us to develop peptide vaccines that are applicable in the majority of the human population. Another approach is to elute MHC class I-binding tumor peptides that will contain as yet unidentified tumor peptides from tumor cells and pulse these peptides onto autologous or MHC class-I-matched allogeneic, APCs. This approach clearly depends on the availability of sufficient, appropriate tumor material and can be applied to cancer patients regardless of the MHC class I phenotype.

Similar vaccination strategies may be applied in various other types of malignancies. For instance, vaccination with HER-2/neu derived peptides, alone, with adjuvant, or pulsed onto APCs, may be considered in HLA-A2 positive breast, ovarian, or lung cancer patients with minimal residual disease. Other attractive candidates for vaccination are HLA-A2-binding peptides derived from HPV E6 and E7 in cervical cancer patients.

Although the recognition of peptide class I complexes is sufficient to trigger target cell lysis, priming of CTL responses requires the presentation of the relevant antigen by professional APCs capable of providing costimulation. At present, vaccination of cancer patients with peptides or tumor proteins loaded onto DCs seems to be one of the most promising approaches. In the past, the use of DCs has been limited by the difficulty of obtaining large numbers of these APCs. Now, DCs can be routinely prepared from peripheral blood leukapheresis samples or buffy coats that are cultured with GM-CSF plus IL-4 [176]. After a few days of culturing with cytokines, DCs can be pulsed or otherwise manipulated in vitro before administration to the patient.

The immunological monitoring of cancer patients will be critical for understanding the nature of the immune responses to vaccination. At present, it remains to be established which of the existing assays is the most accurate in vitro correlate of clinical response. Immunological monitoring assays may include CTL precursor frequency assays, CTL cloning procedures, and TCR analysis on peripheral blood lymphocytes as well as on TILs. Overexpression of TCR may be associated with clonal expansion of antigen-specific T cells and a beneficial immune response to therapy. Analysis of such TILs may be more informative than analysis of circulating blood lymphocytes [174, 177].

Conclusions

Using past experiences, there are now many possibilities for constructing new-generation vaccines which are more rational in the specificity of their composition. Progress in molecular genetics enables us to explore the potential of genetically modified tumor cell vaccines, tumor peptide, protein, or DNA-based tumor antigen vaccines in cancer patients. The value of DCs needs to be established, and new adjuvants need to be explored. Major hurdles for vaccination remain the heterogeneity and variability of human cancer, and the immune escape mechanisms of cancer cells. Decisions regarding type of vaccination strategy should include assessment of tumor antigen and HLA class I expression in the individual patient's tumor.

Results from animal studies and human trials of various vaccine types indicate that active immunization against a patient's preexisting tumor is likely to be effective only when the tumor load is small. Unfortunately, the vast majority of clinical trials has been in patients where the cancer is widespread. Once it has been shown that a new protocol is feasible, patients who are at high risk to develop metastases but who still have minimal residual disease should be selected for vaccination strategies. Immunological monitoring of clinical vaccination trials is critical to our understanding of the complex events that happen in vivo following administration of a vaccine. Therefore, efforts should be made to further develop reliable assays for efficient monitoring of the state of immunization of cancer patients against tumor antigens.

Instead of whole tumor cells, synthetic peptides whose sequences correspond to epitopes of tumor antigens recognized by T lymphocytes can be used as prophylactic vaccines to prevent tumor occurrence in patients at high risk of developing melanoma or as a therapeutic vaccine in patients with metastatic disease.

The admittedly modest successes thus far obtained with vaccine trials in patients nevertheless offer grounds for optimism that important progress in immunotherapy of cancer will be made.

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Melanoma Research, (1995) Vol. 5, No. 6, pp. 443-444

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#3260 Tuesday, April 23, 1996, 1:00–5:00, Poster Section 9

Human melanoma cells can express two plasmid vectors within the same cell following particle-mediated gene transfer. Albertini, M.R.,[1] Emler, C.A.,[2] Schell, K.,[1] Sheehy, M.J.[2] *[1]University of Wisconsin Comprehensive Cancer Center, Madison, WI 53792 and [2]Agracetus, Inc., Middleton, WI 53562, USA.*

T-cell activation requires T-cell recognition of the stimulating antigenic peptide bound to major histocompatibility complex (MHC) molecules and the delivery of a second co-stimulatory signal by the antigen-presenting cell to the T-cell. The purpose of this study was to determine if human melanoma cells could be molecularly modified by particle-mediated gene transfer to augment expression of both HLA molecules and the B7-1 co-stimulatory molecule (CD80) within the same cell. Established and early passage melanoma cells transfected with human IFN-γ cDNA produced IFN-γ (50 to 5000 pg/ml) and had an up-regulation, or *de novo* appearance, of HLA expression. Following combination gene transfer with cDNAs for both IFN-γ and B7-1, 9% to 33% of these cells had *de novo* expression of both HLA-DR and B7-1. In combination gene transfer experiments with cDNAs for both HLA-A2 and B7-1, *de novo* expression of both HLA-A2 and B7-1 was achieved in 10% to 17%. Treatment of the melanoma cells with aphidicolin prior to gene transfer could increase the expression of the transfected genes, and the role of cell cycle position in this enhanced expression is being evaluated. These findings facilitate the evaluation of distinct molecular modifications to stimulate T-cell immunity to human melanoma.

#3261 Wednesday, April 24, 1996, 8:00–12:00, Room 20

T9 glioblastoma cells transduced with the rat Interleukin-4 cDNA can elicit an effective intracranial anti-tumor response resulting in long-term glioma immunity. Graf, M.R.[1], Granger, S.W.[1], Hiserodt, J.C.[2] and Granger, G.[1]. *Departments of Molecular Biology and Biochemistry[1] and Pathology[2], University of California, Irvine, CA*

Murine tumor cells genetically engineered to secrete murine Interleukin (IL)-4 can induce a systemic host immune response when implanted subcutaneously (s.c.) and may lead to the establishment of specific tumor immunity. However, because of the lack of an *in vivo* murine glioma model and the species specificity of the IL-4 molecule, the use of IL-4 secreting glioma cells to elicit an intracranial (i.c.) immune response in syngeneic animals has not been investigated. In the presented study, we transduced Fischer rat T9 glioblastoma cells with a retroviral expression vector containing the rat IL-4 cDNA gene and investigated the ability of these cells to elicit an anti-tumor response in syngeneic rats. Transduced clones were isolated and screened for the expression of the rat IL-4 gene by Northern blot analysis. Clone T9/IL4.B produced the highest level of rat IL-4 mRNA. Furthermore, conditioned media from T9/IL4.B cells upregulated MHC class II expression on rat B-cells. Clone T9/IL4.B did not differ from parental and vector control T9 cells in *in vitro* growth rate or expression of MHC class I, class II and ICAM-1 surface antigens. *In vivo*, lethal gliomas developed from i.c. implantation of 10^5 T9 parental or vector control cells in all animals. In contrast, 47% (7/15) of the animals injected i.c. with 10^5 T9/IL4.B cells survived ($p = 0.0004$). However, pathological analysis revealed that 5 of the 8 animals from the T9/IL4.B group that died had no detectable tumor. Survivors were completely resistant to subsequent i.c. challenges with parental T9 cells. In previous studies, we demonstrated that IL-2 or tumor necrosis factor-α secretion from T9 cells does not alter the lethality of i.c. T9 gliomas. These findings suggest that IL-4 secretion by gliomas may have therapeutic value for the treatment of intracranial tumors.

#3262 Tuesday, April 23, 1996, 1:00–5:00, Poster Section 10

Characterization of a sustained release delivery system for combined cytokine/peptide based vaccination using a fully-acetylated poly-N-acetyl glucosamine matrix. Cole DJ*, Gattoni-Celli S, McClay EF, Nabavi N, Warner SN, Newton D, Woolhiser C, Wilson M*, Vournakis J. *MUSC, Dept. of Surgery* and Hollings Cancer Center, Charleston SC.*

Identification of tumor associated antigens (TAA), and their class I MHC restricted epitopes, now allows for the rational design of peptide-based cancer vaccines. The goal of this study was to characterize *in vitro* a GM-CSF/MART-1 peptide based vaccine utilizing a fully-acetylated poly-N-acetyl glucosamine (p-GlcNAc) matrix for sustained release. P-GlcNAc is a highly purified chitin-based polymer degraded enzymatically by macrophages within 14–21 days, which has passed FDA biocompatibility testing. GM-CSF (200 ug) was dissolved into p-GlcNAc polymer prior to lyophylization. MART-1(27–35) peptide (256 ug in DMSO) was solubilized into post-lyophylization porous matrix. Peptide release was assayed *in vitro* using previously characterized class I MHC restricted Jurkat T-cells (JRT22) expressing MART-1(27–35) specific T-cell receptor. GM-CSF release was assayed via proliferation of M-07E GM-CSF dependent cells. Biologically active MART-1(27–35) peptide presented by T2 cells recognized by JRT22 was released for up to 6 days (>50 ng/ml). Similarly, greater than 1 ug/ml GM-CSF was released over the same period. The mode of administration is a critical component in the design of peptide-based vaccines. A biocompatible system capable of sustained release of biologically relevant levels of cytokine and TAA peptide potentially provides a more effective microenvironment for antigen presentation. This study demonstrates the sustained release of GM-CSF and MART-1(27–35) peptide from P-GlcNAc matrix, providing the basis for future clinical trials.

#3263 Tuesday, April 23, 1996, 1:00–5:00, Poster Section 10

Use of multiple vaccination vectors for the generation of CTL against a model tumor antigen. RS Chamberlain, KR Irvine, SA Rosenberg, and NP Restifo *Surgery Branch, NCI, NIH, Bethesda, MD*

The identification of tumor associated antigens (TAA) has led to the development of multiple novel vaccination vectors. We sought to compare boosting immunization regimens using either the same vaccination vector (homologous), or different vectors (heterologous). Utilizing a β-galactosidase (β-gal) tumor model system, BALB/c mice were immunized initially with either 1) no immunogen, 2) an H-2 L^d β-gal peptide emulsified in IFA (s.c.), 3) whole β-gal protein (s.c.), 4) gene gun immunization with a plasmid encoding the β-gal gene (pCMV/β-gal), 5) recombinant vaccinia virus (rVV) or 6) recombinant fowlpox virus (rFPV) both encoding the gene for β-gal (i.v.). Twenty one days later, mice received a boosting immunization to encompass heterologous and homologous possibilities. Spleens were harvested 4, 6 and 8 days after immunization, and a primary CTL assay was performed. An *in vivo* primary CTL response against a β-gal expressing tumor was elicited by day 4 in only the following groups (50:1 E:T lysis data shown): β-gal DNA boosted with either rVV (31%) or rFPV (64%), β-gal protein boosted with rVV (48%) or rFPV (65%), rVV boosted with rFPV (35%), and rFPV boosted with rVV (67%). No *in vivo* primary CTL was seen with any combination of homologous boosting. Antibody responses were enhanced in all cases in which whole protein was used. Vaccine strategies utilizing heterologous boosting, but not homologous boosting, elicited an *in vivo* primary CTL response. These data suggest that heterologous boosting strategies may have more therapeutic potential for the development of future cancer vaccines.

#3264 Tuesday, April 23, 1996, 8:00–12:00, Poster Section 9

Interleukin-10 enhancement of recombinant viral vaccines in the treatment of a murine tumor. HL Kaufman, J Rao, V Bronte, SA Rosenberg, and NP Restifo *Surgery Branch, NCI, NIH, Bethesda, MD*

Interleukin-10 (IL-10), an 18 kD cytokine produced by T_H2 cells, inhibits cell-mediated inflammation. This cytokine was shown to significantly augment both the reduction in three day old pulmonary metastases and improve survival when administered following a recombinant vaccinia virus encoding the model antigen, β-galactosidase (β-gal) in mice bearing the β-gal expressing tumor line, CT26.CL25.

The potential mechanisms of IL-10 enhancement of vaccinia immunotherapy were examined. No direct *in vitro* anti-tumor activity of IL-10 was seen in a ^{3}H-thymidine proliferation assay. Active treatment utilizing the non-replicating fowlpox virus expressing β-gal followed by IL10 administration yielded similar results to the vaccinia virus, indicating that the primary function of IL-10 was not to enhance viral replication, thus prolonging exposure to antigen. IL-10 resulted in decreased viral titers in ovarian tissue after vaccinia infection, suggesting an enhanced immune response against the recombinant virus as well. This effect was not due to a humoral response, as antibody titers did not rise until after viral titers had become negligible. While the exact mechanism of IL-10 augmentation of recombinant viral immunogens remains unclear, the adjuvant effect of IL-10 may be useful in clinical trials using immunization with recombinant viruses expressing human tumor antigens.

#3265 Tuesday, April 23, 1996, 1:00–5:00, Poster Section 10

Evaluation of viral promoters for use in poxvirus-based anti-cancer vaccines. V. Bronte, M.W. Carroll[¶], M. Wang, T.J. Goletz*, B. Moss[¶], S.A. Rosenberg, and N.P. Restifo. *Surgery Branch, and Metabolism Branch*, NCI, Laboratory of Viral Diseases, NIAID[¶], NIH, Bethesda, MD.*

Clinical protocols are being developed using recombinant poxviruses expressing tumor associated antigens (TAA). The optimal promoters for use in these viruses needs to be elucidated. Poxvirus promoters operating before, or after viral DNA replication are designated early and late promoters, respectively. The most powerful late promoters express 20–50 fold more protein than early promoters. To evaluate the influence of timing and quantity of TAA production on the immune response against the TAA, we used a panel of 12 recombinant vaccinia viruses (rVV) expressing a model TAA, β-galactosidase (β-gal), under the control of various vaccinia promoters. rVV containing the β-gal gene driven by either early or late promoters could prime mice for a CTL response resulting in specific protective immunity against a subsequent challenge with a β-gal-positive tumor (prolongation of mean survival time (m.s.t.) of 2.86 and 2.76 fold for early and late promoters, respectively). However, only the combination of IL-2 and rVV in which strong early promoters were used significantly extended the survival in mice bearing pulmonary metastases (m.s.t. in mice treated with the strongest early rVV was 2.2 fold greater than the control compared to only 1.4 for the best late promoter, $p < 0.05$). Dendritic cells and macrophages, professional APC involved in stimulation of naive T lymphocytes, expressed β-gal only under the control of early promoters suggesting that the successful application of rVV in the active immunotherapy of cancer may require the use of early promoters, able to drive the production of the TAA in infected dendritic cells and macrophages.

#3266 Monday, April 22, 1996, 09:55–10:10, Room 10

Immunization of melanoma patients with melanoma cell vaccine induces anti-MAGE-1 immunity. Okamoto, T., Yuzuki, D., Morton, D. L., Hoon, D. S. B. *John Wayne Cancer Institute, Saint John's Hospital, Santa Monica, CA.*

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Melanoma Research, 3, pp. 51–61

Increased spontaneous mutation rates and prevalence of karyotype abnormalities in highly metastatic human melanoma cell lines

M. Bailly,* S. Bertrand and J.-F. Doré
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Previous studies have suggested that increased malignant potential might be related to increased genomic instability, but this issue still remains controversial. We tested this hypothesis in a human tumour spontaneous metastasis model, using six clones and variants isolated from the parental poorly metastatic M4Be melanoma cell line, and expressing various metastatic abilities. The spontaneous rates of mutation to ouabain resistance measured in these cells by Luria and Delbrück fluctuation analysis correlated with the metastatic ability of the cells: moderately and highly metastatic cells showed spontaneous mutation rates 10 to 50 times higher than those of poorly metastatic cells. Genomic instability at the chromosome level was assessed by searching for accumulated structural abnormalities in the moderately and highly metastatic cell lines. All the cell lines appeared hypertriploid, and showed comparable modal numbers and great chromosome dispersion. Unstable DNA amplification in the form of double minute chromosomes was shown in one of the four poorly metastatic cell lines, and in a significantly higher proportion of the cells of two of the three metastatic cell lines. Abnormal chromosomes were demonstrated in all cell lines, with markers involving specific rearrangements of chromosomes 1, 6, 7, 8, 9, 11, 14 and 15, as frequently observed in human melanoma cells. Clonal markers were present in all cell lines, documenting the common origin of all variants and clones, and specific marker amplification was noticed in highly metastatic cells compared to poorly metastatic lines. These results suggest that human tumour progression might be accompanied both by an increase in genomic instability and by accumulation of karyotypic abnormalities.

Key words: Genetic instability, human melanoma, metastasis, progression.

*To whom correspondence should be addressed.

M. Bailly was supported by a scholarship from the Association pour la Recherche sur le Cancer and is presently a Research Fellow of the Centre Léon Bérard. This work was supported by grants from the Comité de Haute Savoie of the French National League against Cancer, FeGEFLUC, the French Federation of Cancer Centers, and by a grant from the Association pour la Recherche sur le Cancer (ARC grant 6387) to S. Bertrand.

Introduction

Tumour cell progression, especially in terms of invasive and metastatic phenotypes, is a major impediment to successful treatment of disease. The acquisition by neoplastic cells of the capacity to invade locally and to metastasize remains one of the fundamental definitions of malignancy. Since the concept of *in vivo* neoplastic progression was first introduced by Foulds in 1957,[1] and strengthened by Nowell in 1976,[2] several lines of evidence have supported the hypothesis that an increasing incidence of genetic alterations generated by random somatic mutational events is responsible for the heterogeneity and progression of tumour cells to increasingly malignant and less responsive states.[3–6] It is well established that advanced cancers in general show more extensive chromosomal aberrations than do early stage tumours.[7–11] Nowell postulated that clonal evolution of tumour cell populations might result from enhanced genetic instability, which would increase the probability of occurrence of further genetic alterations and their subsequent selection.[2,3] The possible interpretation of such a hypothesis predicts that tumour cells that progress to advanced malignancy would not only show accumulated genomic abnormalities but would also express increased rates of genomic change.[2,12]

Fluctuation analysis, first described in 1943 by Luria and Delbrück to evaluate spontaneous bacterial resistance to virus infection,[13] although suffering from known limitations,[14–16] is a useful technique for analysing aspects of the genomic instability of prokaryotic and eukaryotic cells.[13,17–22] This analysis is based on the following observation: when cells are distributed from a large stock culture into dishes containing a selective agent, there is little variation in the number of mutant colonies isolated from each dish. However, when a series of parallel subclonal cultures is grown up from the stock culture (either from small inocula or ideally from single cells) and transferred to the dishes containing the selective agent, there is substantial variation in the

number of colonies in different dishes. The extent of this variation provides a means of measuring the genomic instability of the cells. The first attempts to measure and compare the mutation rates of normal cells versus malignant cells or the mutation rates of cells at different stages of tumour progression were performed about 10 years ago.[12,15,23-25] Since then, a large body of work has been reported which, unfortunately, provided no general consensus for or against the existence of a direct relationship between mutation rates and progression to more advanced stages of malignancy.[17-22] However, most of these studies used murine tumour cells or human tumour cells in the first steps of malignant transformation and, to the best of our knowledge, none specifically dealt with the measurement of mutation rates and with the acquisition of metastatic ability in human tumour cell populations.

We recently developed in our laboratory a model to study spontaneous metastasis following s.c. injection of human tumour cells into antithymocyte immunosuppressed newborn rats.[26,27] From the original melanoma cell line M4Be, we selected clones and variants with different metastatic abilities. This gave us the opportunity to study the final steps of melanoma cell progression, i.e. invasion and metastasis.[26] We then attempted to compare the inherent genetic instability of these different cell lines as an approach to analyse the mechanisms involved in their progression to the metastatic phenotype. This report describes the measurement of spontaneous mutation rates at the autosomal codominant Na^+/K^+-adenosine triphosphatase (ATPase) locus for four poorly metastatic, one moderately metastatic and two highly metastatic melanoma cell lines. Mutants were selected by resistance to 3×10^{-7} M ouabain in optimal conditions, as previously described.[25] A detailed karyotypic analysis was also performed to search for accumulation of chromosomal abnormalities, arising as a consequence of genomic instability at the chromosome level. We report a good correlation between genomic instability, increased prevalence of specific karyotypic abnormalities, and the spontaneous metastatic ability of the seven cell lines we used in this study.

Materials and methods

Cell lines

The M4Be melanoma cell line has been previously described.[26,27] IC8, P4, G1 and G9 are clones of this cell line which were obtained either by limiting dilution (IC8) or by cloning in semi-solid agar (P4, G9, G1).[26] The 7GP122 variant was selected *in vivo* through eight serial transplantations of lymph node metastases of M4Be cell line into immunosuppressed newborn rats. The T1P26 variant was established in culture after two direct successive transplantations of M4Be tumours into immunosuppressed newborn rats. With the exception of the T1P26 variant, the derivation of variants and clones from the M4Be cell line has been previously described.[26,28] The common origin of all these cell lines is attested by their karyotype and by their sharing common marker chromosomes with the parental cell line M4Be. These cell lines were routinely maintained as monolayer cultures in MacCoy 5A medium (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (Integro-BV, Holland). They are regularly screened for *Mycoplasma* contamination using the Hoechst 33258 fluorescence staining procedure.

Metastasis assay

The assay for spontaneous metastatic ability was performed as previously described.[26,27] Briefly, 10^6 cells in 0.1 ml phosphate buffered saline were injected s.c. into newborn Wistar rats (IFFA CREDO, St-Germain-sur l'Arbresle, France) immunosuppressed with antithymocyte serum, and tumourigenicity and metastatic potential were recorded after 3 weeks.

In vitro growth parameters

Cells were seeded in replicate 60 mm tissue culture dishes (Falcon, Becton Dickinson Labware, UK) at a density of 10^5 cells/dish. Two sample dishes were treated each day with a solution of 0.25% trypsin–0.02% EDTA and the mean number of cells per dish was determined. The logarithmic values of the number of cells were plotted as a function of the time, and the doubling time and the saturation density were determined from the curves at the exponential growth and plateau phases, respectively, for each cell line.

Selection of ouabain-resistant variants

A total of 10^6 M4Be cells was plated in 100 mm tissue culture dishes (Falcon, Becton Dickinson Labware, UK) in MacCoy 5A medium supplemented with 15% foetal calf serum and 3×10^{-7} M ouabain (Fluka AG, Switzerland). They were allowed to grow at 37°C in a humidified atmosphere until colonies were clearly visible. Cells were then harvested using a solution of 0.25% trypsin–0.02% EDTA and plated in 25 cm^2 flasks (Corning, NY, USA) in medium supplemented with 10% foetal calf serum and 3×10^{-7} M ouabain. They were

cultured for two passages in this medium; then medium without ouabain was used for further multiplication before the metastasis assay was performed. These cells will be referred to as M4BeOua^R cells.

Cloning efficiency assay

To determine cloning efficiencies, the cells were plated at 100–1000 cells/100 mm culture dish in 10 ml of cloning medium (MacCoy 5A medium supplemented with 15% foetal calf serum). After 14 days' growth, the colonies were counted following simultaneous fixing and staining with a 20% ethanol–water solution containing 1% Crystal Violet (w/v). Plating efficiency was calculated as: (number of colonies counted on day 14/number of cells originally plated) × 100.

Ouabain cytotoxicity assay

Cells were seeded at various densities from 10^2 to 10^6 cells/dish into several concentrations of ouabain in cloning medium. Control cultures were seeded at 100 cells/dish in the absence of ouabain. All cultures were refed after 7 and 14 days with medium containing the appropriate concentrations of ouabain. After 14 and 21 days, respectively, for the control and the test dishes, the number of surviving colonies per dish was determined following fixing and staining as described above. Relative plating efficiency was estimated as: (% plating efficiency in test medium)/(% plating efficiency in control medium).

Fluctuation analysis

A fluctuation assay was developed by adapting the technique previously described by Elmore and Barrett.[25] Parameters were determined in preliminary experiments according to the method of Fu *et al.*[14] Replicate platings of 100–1000 cells were made into each of 24 tissue culture dishes (100 mm) in 10 ml cloning medium. Each culture was refed with fresh medium after 7 days. After 14 days, four randomly selected dishes were fixed and stained, and the average number of colonies per dish (N_0) was determined. At the same time, the colonies in the remaining dishes were redistributed by treating the cells for 5 min with a solution of 0.25% trypsin–0.02% EDTA. Fresh cloning medium was then added to the dishes, and they were allowed to grow. At periodic intervals thereafter, two dishes were trypsinized and the mean cell number was determined. When a final cell number of approximately 10^6 cells/dish was reached, six of the remaining dishes were harvested and reseeded each in 10 tissue culture dishes (100 mm) in 10 ml of selective cloning medium (cloning medium supplemented with 3×10^{-7} M ouabain), so that a total of 60 dishes was obtained. All dishes were refed with selective cloning medium on days 7 and 14, and the resultant ouabain-resistant colonies were fixed, stained, and counted after 21 days of growth.

Estimation of spontaneous mutation rate

The spontaneous mutation rate, a, was calculated using both the method of means using equation (8) of Luria and Delbrück:[13]

$$r = aN_t \ln(CaN_t)$$

modified by Capizzi and Jameson,[29] and P_0 method,[13]

$$a = -\ln(P_0)/N_t$$

where r is the mean number of mutant colonies per parallel culture, N_t is the final cell number at the time of ouabain application, C is the number of parallel cultures, and P_0 is the fraction of cultures without mutant colonies.

Karyotype analysis

Chromosomal analyses were performed using the R-banding technique, which has been previously described.[30] Modal numbers were determined from a minimum of 50 mitoses and 6–39 karyotypes were classified for each cell line.

Results

Metastatic ability and growth parameters of M4Be melanoma cell line and derived clones and variants

The origin, tumourigenicity and metastatic ability of the different cells are described in Table 1. IC8, P4 and G9 clones, like the M4Be cell line, are poorly metastatic, and G1 clone has a significantly higher but moderate metastatic potential, while 7GP122 and T1P26 are highly metastatic variants. Representative pulmonary samples recovered 3 weeks after subcutaneous injection of M4Be, IC8, 7GP122 and T1P26 cell lines are shown in Figure 1. These cells present comparable doubling times and saturation densities, these parameters being not obviously related to the metastatic ability of the cell lines (Table

Table 1. Tumourigenicity and metastatic potential in rats of M4Be cell line and derived clones and variants

	Cell origin	Tumour incidence[a]	Mean tumour weight ± SD (mg)	Axillary lymph node involvement incidence (%)[b]	Pulmonary metastasis incidence (%)[c]	Lung nodules/rat	
						Median	Range
M4Be	Lymph node metastasis	14/14	524 ± 515	57	36	0	0–30
IC8	*In vitro* selected clone	34/34	305 ± 413	44	12	0	0–100
P4	*In vitro* selected clone	11/12	43 ± 32	0	8	0	0–5
G9	*In vitro* selected clone	22/22	70 ± 93	77	68	2	0–50
G1	*In vitro* selected clone	16/16	591 ± 346	50	81	17	0–>250[d]
7GP122	*In vivo* selected variant	27/27	1466 ± 1131	11	89	100	0–⩾300[e]
T1P26	*In vivo* selected variant	21/21	1208 ± 1033	90	100	>250	2–⩾300[e]
M4BeOua^R	Ouabain-resistant variant	12/12	Unmeasurable trace	0	0	0	0

[a]Number of animals with tumour/number injected.
[b]Percentage of animals with axillary lymph node involvement.
[c]Percentage of animals with lung metastasis.
[d]Distribution statistically different from that of M4Be ($p = 0.01$, Wilcoxon test).
[e]Distribution statistically different from that of G1 ($p < 0.01$, Wilcoxon test).

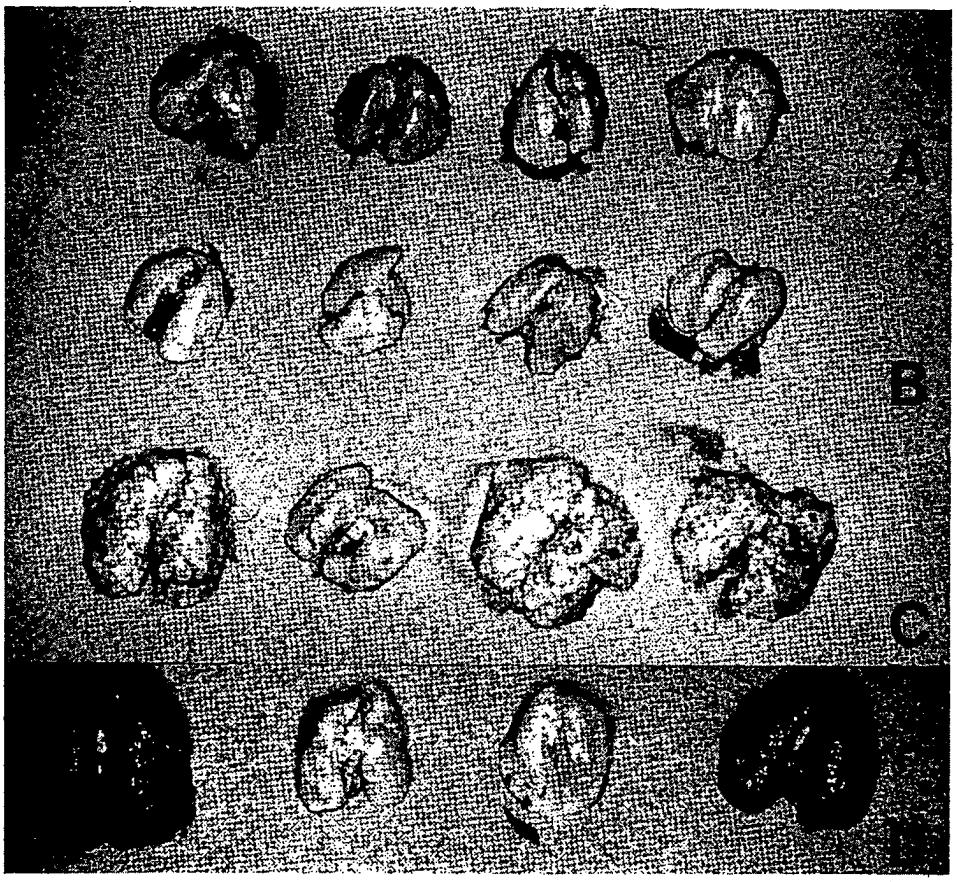

Figure 1. Representative pulmonary samples of 21-day-old immunosuppressed rats grafted subcutaneously with 10^6 melanoma cells on day 0; A, M4Be poorly metastatic cell line B, IC8 low metastatic clone; C, 7GP122 highly metastatic variant; D, T1P26 highly metastatic variant.

Table 2. Growth parameters of M4Be cell line and derived clones and variants

	Doubling time (h)	Saturation density (cells/cm²)	Plating efficiency (% ± SD)
M4Be	29	2.2×10^5	43 ± 10
IC8	26	1.1×10^5	64 ± 18
P4	ND[a]	ND	22 ± 3
G9	29	1.3×10^5	31 ± 12
G1	16	2.7×10^5	11 ± 13
7GP122	23	3.0×10^5	62 ± 7
T1P26	25	1.9×10^5	21 ± 19

[a]Not done.

2). The plating efficiency of the different cells in the absence of selective medium varied from 11 to 64%, again without any apparent relationship with the metastatic ability (Table 2).

Ouabain cytotoxicity

Figure 2 shows the cytotoxic effect of ouabain on M4Be cell line and derived clones and variants, as measured by the relative plating efficiency of the cells as a function of ouabain concentration: poorly metastatic, and moderately and highly metastatic cells are depicted on Figures 2A and 2B respectively (the case of M4BeOua^R variants will be discussed below). The general aspect of the different curves was similar for each cell line examined, and in all these cells, ouabain-resistant mutants appeared to accumulate at a concentration of about 3×10^{-7} M ouabain (plateau phase). The relative plating efficiencies at the plateau phase varied from one cell line to the other (from 10^{-7} for the G9 clone to about 2×10^{-6} for T1P26 variant), but did not appear to be directly related to the metastatic ability of the cell lines. Indeed, they were quite similar to that of M4Be parental cell line, except for T1P26 (Figure 2B).

Measurement of spontaneous mutation rates

The estimated ouabain mutation rates of the different cell lines are presented in Table 3. These mutation rates were comparable for the four poorly metastatic cell lines, and ranged from 0.44 to 2.76×10^{-7} and 0.21 to 3.19×10^{-7} mutations/cell/generation respectively, when the mean method or the P_0 method were employed for calculation. The mean values calculated from the three estimations given by the two methods of calculation are $1.09 \pm 0.71 \times 10^{-7}$ for M4Be cells, $1.615 \pm 0.95 \times 10^{-7}$ for clone IC8, $2.02 \pm 1.08 \times 10^{-7}$ for clone P4 and $1.005 \pm 0.65 \times 10^{-7}$ for clone G9. With the exception of the results of experiment 1, the mutation rates estimated for the moderately metastatic G1 clone were about 10 times higher than that of M4Be cells (mean value for the three experiments: $11.09 \pm 8.71 \times 10^{-7}$, $p < 0.05$ with Student's t test). The values obtained for the highly metastatic variants were similar to that of the G1 clone for the lowest, and up to four times higher for the highest ($7.81–80 \times 10^{-7}$ with the method of the means, and $7.17–23.5 \times 10^{-7}$ with the P_0 method). The

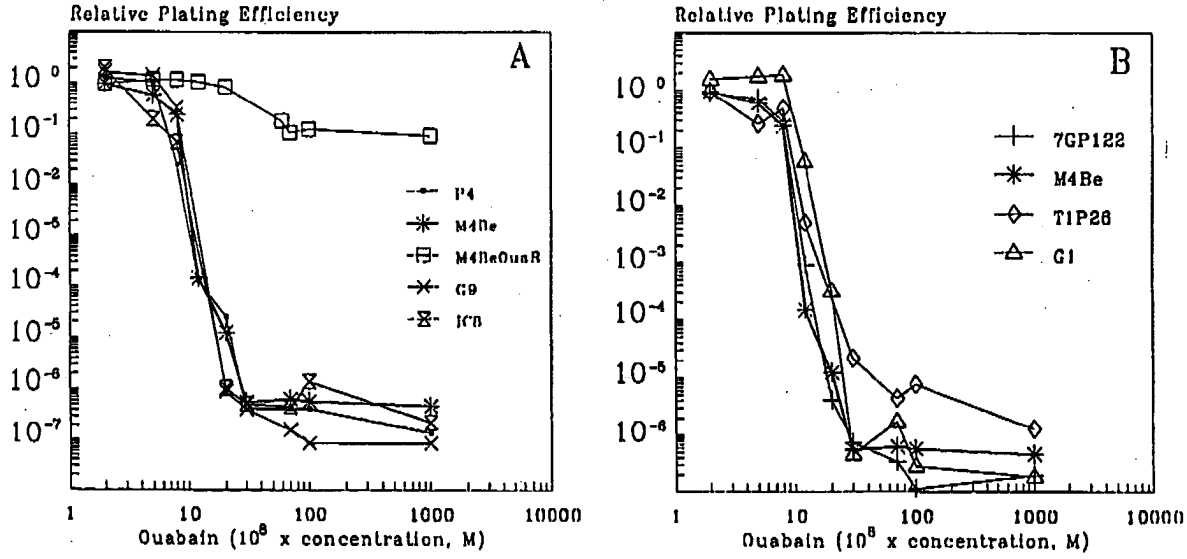

Figure 2. Relative plating efficiency of melanoma cells in the presence of graded ouabain concentration. Each point represents the mean of 20–30 replicate dishes. A: M4Be, IC8, P4, and G9 poorly metastatic cells, and M4BeOua^R variant. B: M4Be G1, 7GP122 and T1P26 moderately and highly metastatic cells.

Table 3. Estimated mutation rates of M4Be cell line and derived clones and variants

	Experiment 1	Experiment 2	Experiment 3
M4Be			
$N_0 \pm SD$[a]	86 ± 16	96 ± 14	40 ± 3
$N_t \pm SD$ ($\times 10^{-6}$)[b]	0.97 ± 0.35	3.37 ± 0.40	3.50 ± 2.40
C[c]	55	58	42
$r \pm SD$[d]	0.04 ± 0.28	0.81 ± 2.73	2.45 ± 3.70
No. of cultures without mutant colonies	54	41	21
P_0	0.98	0.71	0.50
Mutations/cell/generation ($\times 10^7$)[e]			
Mean method	0.44	0.85	2.06
P_0 method	0.21	1.02	1.98
IC8			
$N_0 \pm SD$	45 ± 10	70 ± 33	97 ± 6
$N_t \pm SD$ ($\times 10^{-6}$)	3.39 ± 1.18	2.52 ± 1.94	1.12 ± 0.45
C	59	59	40
$r \pm SD$	0.88 ± 1.15	0.31 ± 0.92	0.60 ± 1.30
No. of cultures without mutant colonies	30	49	28
P_0	0.51	0.83	0.70
Mutations/cell/generation ($\times 10^7$)			
Mean method	0.90	0.57	2.30
P_0 method	1.99	0.74	3.19
P4			
$N_0 \pm SD$	61 ± 46	61 ± 46	178 ± 19
$N_t \pm SD$ ($\times 10^{-6}$)	1.51 ± 1.24	4.97 ± 2.51	1.00 ± 0.52
C	42	57	57
$r \pm SD$	0.17 ± 0.81	6.07 ± 7.11	0.70 ± 1.57
No. of cultures without mutant colonies	40	15	42
P_0	0.95	0.26	0.74
Mutations/cell/generation ($\times 10^7$)			
Mean method	0.72	2.76	2.60
P_0 method	0.32	2.68	3.06
G9			
$N_0 \pm SD$	161 ± 54	261 ± 13	265 ± 31
$N_t \pm SD$ ($\times 10^{-6}$)	0.84 ± 0.26	1.21 ± 0.41	3.79 ± 0.86
C	58	55	52
$r \pm SD$	0.16 ± 0.36	0.25 ± 0.64	0.08 ± 0.27
No. of cultures without mutant colonies	49	46	48
P_0	0.84	0.84	0.92
Mutations/cell/generation ($\times 10^7$)			
Mean method	1.10	1.07	0.17
P_0 method	2.00	1.47	0.22
G1			
$N_0 \pm SD$	161 ± 29	165 ± 23	171 ± 19
$N_0 \pm SD$ ($\times 10^{-6}$)	4.23 ± 2.11	1.26 ± 0.49	1.21 ± 0.39
C	53	40	55
$r \pm SD$	0.57 ± 3.68	10.27 ± 13.20	10.72 ± 9.16
No. of cultures without mutant colonies	50	15	4
P_0	0.94	0.37	0.07
Mutations/cell/generation ($\times 10^7$)			
Mean method	0.54	18.05	18.40
P_0 method	0.13	7.79	21.64
7GP122			
$N_0 \pm SD$	100 ± 9	169 ± 27	170 ± 18
$N_t \pm SD$ ($\times 10^{-6}$)	2.11 ± 1.02	4.68 ± 1.49	2.30 ± 1.00
C	59	59	58
$r \pm SD$	7.54 ± 16.88	45 ± 45	107 ± 52
No. of cultures without mutant colonies	13	0	0
P_0	0.22	—	—
Mutations/cell/generation ($\times 10^7$)			
Mean method	7.81	15.8	68.4
P_0 method	7.17	—	—

(continued)

Table 3. *(continued)*

	Experiment 1	Experiment 2	Experiment 3
T1P26			
$N_0 \pm$ SD	99 ± 33	39 ± 2	36 ± 2
$N_t \pm$ SD ($\times 10^{-8}$)	1.53 ± 0.44	2.13 ± 0.87	0.63 ± 0.25
C	17	47	22
$r \pm$ SD	40.5 ± 36.3	23.4 ± 23.2	23.8 ± 31.1
No. of cultures without mutant colonies	0	9	5
P_0	—	0.19	0.23
Mutations/cell/generation ($\times 10^7$)			
Mean method	53.6	20.6	80.0
P_0 method	—	7.8	23.5

[a]Mean initial cell number
[b]Mean final cell number
[c]Number of parallel cultures
[d]Mean number of mutant colonies per culture
[e]See Material and Methods for calculation

P_0 method was less informative in these cases because the proportion of the cultures without any mutant colonies was very low and was zero in most experiments. The mean values obtained for the highly metastatic variants 7GP122 and T1P26 were 20 and 40 times higher, respectively, than that of M4Be cells ($24.795 \pm 25.40 \times 10^{-7}$, $p = 0.1$ for 7GP122 variant and $37.1 \pm 26.18 \times 10^{-7}$, $p < 0.02$ for T1P26 variant).

As the estimated mutation rates appeared to be grossly correlated with the metastatic potential of the different cell lines, we attempted to determine whether ouabain resistance could be involved in the expression of the metastatic phenotype, and whether our results could be due to a direct relationship between a mutation at the ATPase locus and the expression of metastatic ability. Hence, M4BeOua^R ouabain-resistant variant was selected from the M4Be cell line. M4BeOua^R responded to increasing ouabain concentration in a similar way to that of the other cell lines, but presented a plateau phase at about 10^{-1} (relative plating efficiency, Figure 2A). The *in vivo* behaviour of M4BeOua^R cells, shown in Table 1, shows that ouabain resistance has no direct causal effect on metastatic ability: M4BeOua^R was even less metastatic than the poorly metastatic M4Be cell line. M4BeOua^R cells had even lost their ability to form detectable tumours after s.c. injection, though they exhibited *in vitro* growth parameters similar to that of M4Be cells (data not shown).

Karyotype analyses

Spontaneous mutation rate studies provided us with results showing a correlation between the genomic instability and the metastatic ability of the cell lines. We then attempted to expand our studies by seeking possible alterations at the chromosome level, where changes could have accumulated with a higher frequency in the highly metastatic cell lines. For this purpose, we performed a comparative analysis of the prevalence of cytogenetic structural abnormalities in the different cell lines.

All cell lines were hypertriploid, showing quite comparable modal numbers (Table 4). Chromosome dispersion was quite high, ranging from 34 to 153 chromosomes per mitosis, the cell line expressing the narrowest chromosome range being the 7GP122 variant. Comparative analysis of abnormal marker chromosomes in M4Be and derived cell lines showed a possible relationship between accumulation of karyotype abnormalities and metastatic ability. Nineteen different markers have been found among the different cell lines studied here (Figure 3). Their detailed cytogenetic characterization and their respective frequencies are presented in Table 5. More than half of the normal chromosomes were involved in marker formation, with the exception of chromosomes 10, 17, 18, 19, 20, 21, 22, 23, X and Y. The chromosome most frequently involved in rearrangements was chromosome 7, and the other chromosomes regularly involved in the constitution of markers were chromosomes 1, 3, 6, 9, 12 and 15. Five clonal markers appeared to be characteristic of M4Be cells and the derived sublines, markers M1, M2, M3, M7 and M13, respectively derived from chromosomes 14 and 11, 7 and 8, 9 and 15, 6, and 9 and 15 again. Marker M3 was found much more often in all clones and variants than in M4Be cell line where it appeared not be clonal. Eleven new markers appeared in M4Be-derived cell lines which were not detectable in M4Be cells: M4, M9, M11, M12, M15, M16, M17, M19, M20, M21 and M23. Marker M1 was

Table 4. Modal numbers of M4Be cells and derived clones and variants

	Modal number	Chromosome range
M4Be	74–78	70–153
IC8	75–82	57–103
P4	77–80	49–109
G9	64–75	46–137
G1	67–72	45–142
7GP122	71–75	34–75
T1P26	64–74	47–138

lost in G1, 7GP122 and T1P26 cells, while markers M2, M6 and M8 were found in higher proportions of these cells compared with the four other cell lines. Markers M16, M19, M20 and M23 were exclusively expressed in cells with high metastatic ability, though usually in a sporadic manner.

DNA amplification in the form of double minute (DM) chromosomes was noticed in one out of the four low metastatic cell lines with a low frequency, only 4% of the cells having a unique DM marker (Table 6). Two out of the three metastatic cell lines presented DMs, in 54 and 21% of G1 and 7GP122 cells, respectively, with a number of copies per cell varying from one to two.

Discussion

We measured the mutation rates in seven human melanoma cell lines of low, moderately or high spontaneous metastatic ability after s.c. injection into immunosuppressed newborn rats.[26] This demonstrated a correlation between the estimated spontaneous mutation rates and the spontaneous metastatic ability of the different cell lines. The poorly metastatic cells had estimated mutation rates of about 10^{-7} mutations/cell/generation, while the mutation rates estimated for the moderately and highly metastatic cells were at least one order of magnitude higher ($1–8 \times 10^{-6}$ mutations/cell/generation). The previously reported spontaneous mutation rates for ouabain ranged from 0.2 to 13×10^{-7} mutants/cell/generation for human cells[15,25] and from 0.1 to 8.8×10^{-7} mutations/cell/generation for rodent cells.[12,17,18,20] Elmore *et al.* reported mutation rates of $1.38–8.5 \times 10^{-7}$ and 6 13×10^{-7} mutations/cell/generation for normal and chemically transformed human fibroblasts respectively;[15] these mutation rates are comparable to those reported here for low and moderate metastatic cells.

Plating efficiency, which could be of great importance to the validity of the test,[16,25] sometimes varied greatly between cell lines; however, it is unlikely that this would

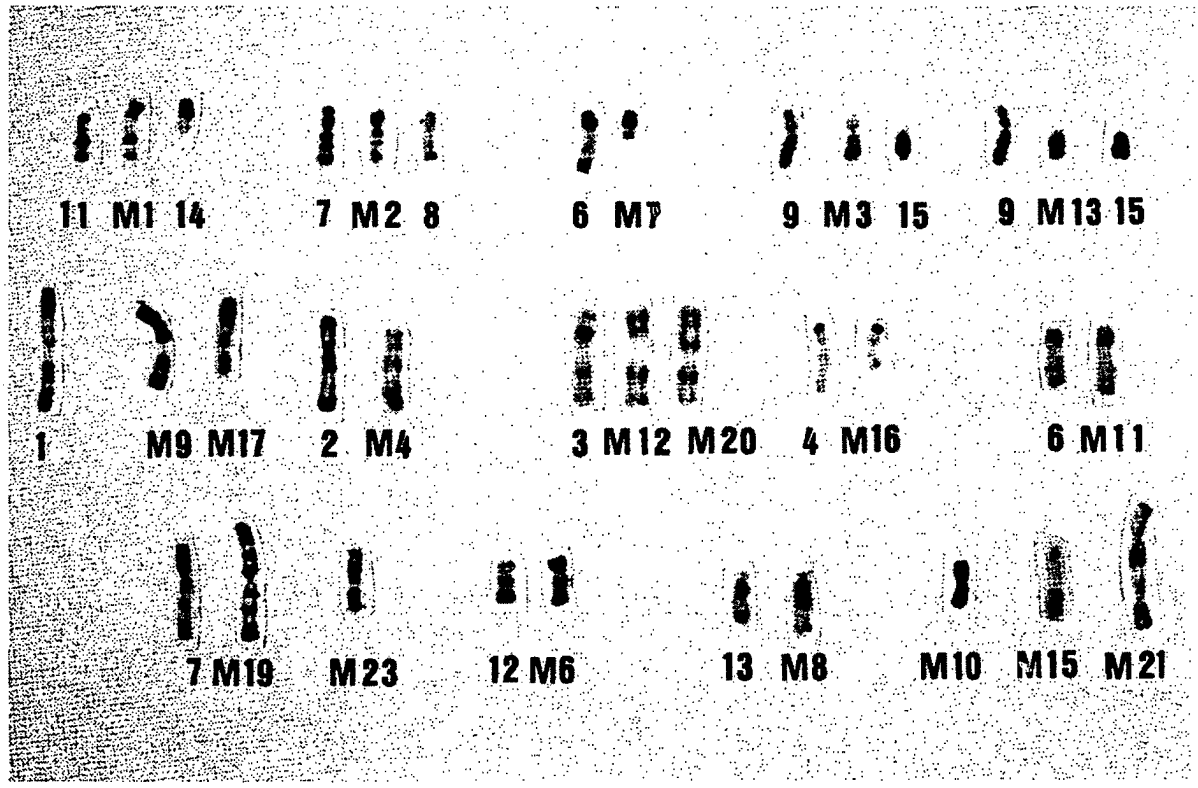

Figure 3. Abnormal marker chromosomes (M) in M4Be cell line and derived clones and variants. Normal chromosomes from which they originated are shown beside the markers.

Table 5. Abnormal chromosomes (markers[a]) in M4Be cell line and derived clones and variants

	Markers																		
Cells	M1	M2	M3	M4	M6	M7	M8	M9	M10	M11	M12	M13	M15	M16	M17	M19	M20	M21	M23
M4Be	++c[b]	++c	±	−	±	++c	+	−	±	−	−	+c	−	−	−	−	−	−	−
IC8	++c	±	++c	−	−	++c	−	+c	−	−	−	+c	−	−	−	−	−	−	−
P4	++c	++c	+c	−	−	++c	−	±	±	−	−	++c	−	−	−	−	−	−	−
G9	++c	++c	++c	(±)	(±)	++c	(±)	(±)	±	(±)	±	+c	(±)	−	(±)	−	−	(±)	−
G1	+c	+++c	++c	−	±	++c	−	±	±	(±)	±	+c	−	(±)	(±)	(±)	(±)	−	(±)
7GP122	+c	+++c	++c	−	±	++c	±	−	−	(±)	(±)	+c	(±)	±	−	−	±	−	−
T1P26	+c	+++c	++c	−	±	++c	±	−	−	−	−	++c	−	+c	±	−	−	−	−

[a]M1 = t(11;14) (p15.3;q24); M2 = t(7;8) (p14;p21); M3 = t(9;15) (q21?:q13?); M4 = t(2;5?) (q;p?) M6 = t(12;?) (p12;?); M7 = del(6)(q11 q24); M8 = dup(13) (q13-q21.1?); M9 = del(1)(p34); M10 = not identified; M11 = dir.ins(6) (q21); M12 = t(3;?)(p14;?); M13 = t(9;15) (q13;q21); M15 = not identified; M16 = t(4;12) (p;p); M17 = t(1;?) (q11;?); M19 = iso7q; M20 = t(3;?) (q11;?); M21 = dicentric; M23 = t(7?;16?) (p14?;p12?).

[b]Marker distribution: −, not found; (±), sporadically found; ±, found in a noticeable proportion of the cells; +, found in most of the cells in one copy; ++, found in most of the cells in one or two copies; +++, found in most of the cells in two copies or more; c, clonal marker.

Table 6. DM[a] chromosomes quantitation in M4Be cell line and derived clones and variants

	% mitoses with DM	DM/cell		
		0	1	2
M4Be	0	100[b]	0	0
IC8	0	100	0	0
P4	0	100	0	0
G9	4	96	4	0
G1	54	46	41	13
7GP122	21	79	14	7
T1P26	0	100	0	0

[a]Double minute
[b]Percent of cells

exert a significant influence on our results: a low plating efficiency would result in incomplete recovery of the mutants, and underestimation of the mutation rate. This is, however, not of great significance in our study since the G1 cells which expressed the lowest plating efficiency showed one of the highest values for estimated spontaneous mutation rate. Growth parameters were also comparable for the seven cell lines, and did not appear to interfere directly with the measurement of spontaneous mutation rates. Chromosome dosage can also be an important parameter in fluctuation analysis since chromosomal ploidy level may influence the frequency of mutants.[25,31] If codominant mutations such as ouabain resistance are being studied, hyperploid cells or cells with multiple gene copies are likely to show a higher mutation level when compared with others expressing fewer karyotypic abnormalities. Na^+/K^+-ATPase subunit genes are mainly mapped to human chromosome 1; their minor subunits map to chromosomes 19, 13, or 4.[32] Amplification of chromosome 1 or of either chromosomes 19, 13 or 4 in metastatic cells could account for the increased mutation rates detected. This was not the case since no extra copies of these chromosomes were found in the metastatic G1, 7GP122 and T1P26 cell lines when compared with the four poorly metastatic cells (data not shown). Our results are thus unlikely to be an artefact due to specific karyotypic amplification in the metastatic cells.

To the best of our knowledge, the present work is the only study of the relationship between metastatic ability and genetic instability in terms of spontaneous mutation rates, in sublines derived from a single human melanoma cell line. Warren *et al.* reported a 10- to 15-fold higher mutation rate in fibroblasts from Bloom's syndrome compared with that in fibroblasts of normal individuals.[24] Seshadri *et al.* demonstrated lower mutation frequencies and spontaneous mutation rates for 6-thioguanine resistance in normal lymphocytes compared with malignant cells of two Burkitt's lymphomas and one acute lymphoblastic leukemia T cell line.[21] Unfortunately, it is difficult to compare these results directly with ours since the cell lines and normal lymphocytes used in these studies originated from different donors, while the clones and variants we use all derived from the same cell line. This may minimize possible artefacts due to the different origin of the cells. Elmore *et al.* were not able to demonstrate any significant difference between mutation rates at two independent loci in normal and chemically transformed human fibroblasts.[15] More studies have used rodent cells: some have shown a good correlation between increasing spontaneous mutation rates and metastatic potential;[12,19,22] others have demonstrated no relationship between those two parameters.[17,18,20] Even more puzzling are the results presented by Yamashina and Heppner who reported no relationship between spontaneous mutation rates and metastatic potential, but a correlation between the

frequency of induced mutations and the metastatic potential in mouse mammary tumour cell lines.[17] Our results present evidence that increasing metastatic ability might be related to an increase in spontaneous mutation rates in human melanoma cell lines. We used cloned as well as uncloned cells, and variant cells isolated either rapidly (T1P26) or after numerous *in vivo* passages (7GP122), and all these cell lines expressed a stable metastatic phenotype.[26] It is thus unlikely that the differences we obtained could be attributed to a difference in the stability of the cell lines, especially between clones and variants, as suggested by Kendal *et al.*[20]

In control experiments during fluctuation analyses, we selected a mutant of M4Be cell line (M4BeOua^R) resistant to a concentration of 3×10^{-7} M ouabain. Comparable mutants of human cells have previously been successfully selected using 2×10^{-7} M ouabain.[25] The dramatic loss of tumourigenicity and metastatic potential of this variant compared to M4Be cell line was surprising. Similar results have been obtained by other groups while selecting ouabain or 6-thioguanine-resistant mutants.[33,34] However, we were not able to establish whether this loss of malignancy was directly related to the effect of ouabain, or whether it resulted from an undirect effect of the drug as demonstrated for a 6-thioguanine resistant variant.[33]

The development of ouabain resistance is generally considered to be the result of a single point mutation at that locus. The contribution of such a mechanism to tumour progression, even at rates of about 10^{-6} mutations/cell/generation, might be questioned in view of recent results showing the multiple and rapid genetic changes accompanying the progression of specific tumours.[7,10] Other events, such as gene amplification, might occur at much higher rates.[22] However, increased rates of somatic point mutations at the Na^+/K^+-ATPase locus presumably indicate that point mutations might occur more frequently at loci related to malignant traits. It has recently been demonstrated that point mutations on specific genes could represent essential steps in carcinogenesis and human tumour cell progression.[35,36] Thus, an increase in spontaneous mutation rates at the ouabain locus might reflect a more general increase of the rates of overall genetic changes, and might facilitate the generation and stabilization of unstable genotypic changes required for tumour progression.[5] The results of the karyotypic analysis we performed on the different cell lines indeed support this hypothesis. We thus demonstrated that highly metastatic cell lines have accumulated additional chromosomal abnormalities when compared to poorly metastatic cell lines. Thus, markers M2 [t(7;8)(p14;p21)], M6 [t(12;?)(p12;?)], M8 [dup(13)(q13-q21.1?)] and M11 [dir. ins(6)(q21)] were present at a higher frequency in G1, 7GP122 and T1P26 cells when compared to poorly metastatic cells. In addition, four markers [M16, t(4;12)(p;p); M19, iso7q; M20, t(3;?)(q11;?); and M23, t(7?;16?)(p14?p12?)] were exclusively expressed in cells with high metastatic potential. Unstable DNA amplification in the form of DM chromosomes was also documented in our study. We demonstrated the presence of DMs in two of the three cell lines with high metastatic potential, whereas only one of the four cell lines with low metastatic potential, clone G9, presented only a small percentage of mitoses with a single DM chromosome. The exact nature and role of the sequences amplified in these chromosomes have still to be determined, but they represent one way of expression of the cellular genomic instability.

In conclusion, we have demonstrated that expression of high metastatic ability in human melanoma cell lines is associated with an increase in genetic instability measured by spontaneous mutation rates at ouabain resistance locus. Such genomic instability might account more generally for the accumulation of structural abnormalities and unstable DNA amplification observed in the highly metastatic cells.

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Thanks,

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2923

Enhancement of cell-mediated immunity in melanoma patients immunized with murine anti-idiotypic Mabs (MELIMMUNE®). Pride, M.W., Shuey, S., Grillo-Lopez, A., Murray, J.L. UT M.D. Anderson Cancer Center, Houston, TX 77030 and IDEC Pharmaceuticals, LaJolla, CA.

MELIMMUNE-1 and MELIMMUNE-2 (MELIMMUNE) are murine anti-idiotypic Mabs (Ab2) which mimic separate epitopes on the high molecular weight melanoma-associated proteoglycan. Peripheral blood mononuclear cells (PBMC) obtained from 15 resected Stage I-IV high risk melanoma patients immunized with MELIMMUNE plus the adjuvant SAF-m were tested for in vitro proliferation to various Ab2 [F(ab')2] along with cytotoxicity against ^{51}Cr-labeled target cells. Significant in vitro proliferative leukocyte responses were obtained 4 weeks post immunization when stimulated with F(ab')$_2$ fragments of MELIMMUNE-1 or-2 but not from an isotype control Mab. Preimmune samples demonstrated no in vitro proliferation to the same stimuli. PBMC samples obtained pre and post immunization were also stimulated in vitro with either MELIMMUNE-1 or-2 and IL-2 and tested against the NK-sensitive target line K562, HLA-A2$^+$ melanoma cell line A375 and a HLA-A2$^+$ ovarian cell line. 9/15 patients did not demonstrate significant killing to any tumor target (pre or post immunization) while 3/15 samples demonstrated NK activity which was enhanced post immunization. More significantly, 3/15 patients (HLA-A2$^+$) lysed only the HLA-A2$^+$ melanoma cell line A375. These results indicate that MELIMMUNE enhances cell mediated immune responses (proliferative and cytotoxicity) in melanoma patients by specific and nonspecific mechanisms.

2924

Effect of sialyl Tn configuration and presentation in KLH conjugate vaccines on specificity of the resulting antibody response. [a]Zhang, S.L., [a]Walberg, L.A, [b]Ogata, S., [b]Itzkowitz, S.H., [c]Koganty, R., [c]Reddish, M., [c]Longenecker, B.M., [a]Lloyd, K.O. and [a]Livingston, P.O., [a]Memorial Sloan-Kettering Cancer Center, New York, [b]Mount Sinai School of Medicine, New York, [c]Biomira Inc., Edmonton, Canada.

Sialyl Tn (sTn) is a mucin-associated carbohydrate antigen expressed in most types of human adenocarcinoma. In this study, we compared the immunogenicity of synthetic single sTn disaccharide epitopes and clusters of 3 sTn epitopes covalently linked to KLH through a serine backbone. Ten CB6F1 mice per group were immunized with 30 ug sTn-KLH (epitope ratio 3000/1) or 30 ug sTn(Cluster)-KLH (epitope ratio 30/1) 3 times at 1 week intervals with immunological adjuvant QS-21. Sera drawn after the immunizations reacted with synthetic sTn- and sTn(Cluster)-HSA at median titer of 1:7680 and 1:960 respectively, with ovine submaxillary mucin (OSM, a natural source of sTn) at median titer of 1:10240 and 1:7680 respectively. All sera in a cellular ELISA assay reacted with the sTn-positive human colon cancer cell line LS-C and not with sTn(-) LS-B. Although reactivity of sTn and sTn cluster immune sera (and a series of monoclonal Abs) with sTn expressed by OSM and tumor cells appeared similar, inhibition assays demonstrated different specificities. Reactivity of sTn cluster immune sera and mAbs B72.3 and B239.1 for OSM were inhibited by sTn(Cluster)-HSA but not sTn-HSA. Reactivity of sTn immune sera, however, were inhibited exclusively by sTn-HSA but not by sTn(Cluster)-HSA. These results suggest that sTn is recognized at the tumor cell surface in 2 different configurations. Supported by NIH grant CA 33049.

2925

Active immunotherapy with polyvalent melanoma cell vaccine reduces the incidence of secondary brain metastases in stage IV melanoma. Morton, D.L., Nizze, J.A., Famatiga, E., Foshag, L.J., Wanek, L.A., Dagleish, A. John Wayne Cancer Institute, Santa Monica, CA 90404.

Our polyvalent melanoma cell vaccine (MCV) induces cell-mediated immunity to melanoma-associated antigens on autologous melanoma cells and prolongs the survival of patients with advanced-stage melanoma from 7.5 to 23 months. High responders in CMI assays such as cytotoxic T lymphocyte (CTL)-mediated lysis of melanoma cells had a threefold increase in 5-year survival compared with low responders (*Cancer Res* 1994;54:3342). We hypothesized that one mechanism of MCV's effect was the inhibition of blood-borne metastasis. Since brain metastases are a frequent cause of death in melanoma and since 75-80% of melanoma patients have cerebral metastases at death, we examined the incidence of secondary brain metastases in patients whose initial distant metastatic site was noncerebral. We compared the frequency of brain metastases at the time of death in patients receiving MCV versus chemotherapy or non-MCV immunotherapies. The overall incidence of secondary brain metastases was 49% (262/533) without MCV versus 24.5% (27/110) with MCV ($p<.001$); corresponding median survivals were 7.5 and 16.8 months. These differences were seen at all initial metastatic sites: liver/bone 34.2% (53/155) versus 8.3% (1/12); lung 57% (134/235) versus 30.8% (16/52); skin/lymph nodes/GI 52.4% (75/143) versus 21.7% (10/46). We conclude that MCV alters the metastatic phenotype of melanoma and that this alteration is a major reason for MCV's therapeutic effect.

2926

Systemic immunity in melanoma patients treated with autologous ultraviolet-B irradiated whole tumor cell vaccine with DETOX™. Kharkevitch DD, Eton O, Ross MI, Korostelev AA, Gianan MA, Mansfield PF, Itoh K*, Benjamin RS, Balch CM. UT MD Anderson Cancer Center, Houston, TX, 77030, and *Kurume University School of Medicine, Kurume, Fukuoka 830, Japan.

Induction of autologous tumor-specific immunity is essential for prevention and treatment of metastatic melanoma with whole tumor cell vaccines. The aim of this study is to detect and characterize induction of cellular immunity in melanoma patients (pts) receiving ultraviolet-B irradiated (UVR) autologous whole tumor cell vaccine with DETOX™. Twenty-eight pts with metastatic melanoma have been immunized with 10^7 X-irradiated UVR-tumor cells plus DETOX™ at 2 week intervals x 6 and monthly thereafter. The dynamics over time of lymphokine production by, and cytotoxicity of unstimulated and IL-2 stimulated peripheral blood mononuclear cells (PBMC) are being studied in response to autologous tumor, allogeneic melanomas, tumor of non-melanoma origin and NK-sensitive K-562. To-date five pts treated over a year have developed a cytotoxic response of unstimulated PBMC to autologous tumor. In three of these pts antibody-blocking experiments have revealed that the cytotoxicity was mediated by CD3+CD8+T cells and was abrogated by anti-MHC class I monoclonal antibodies. This ongoing study may help to establish reliable assays to measure the systemic effect of tumor vaccine therapy.

2927

Development and characterization of recombinant adenoviruses Ad2CMV-MART1 and Ad2CMV-gp100 as antigen-specific vaccines for cancer therapy. Zhai,Y., Kawakami,Y., Wadsworth, S.C.*, Cardoza, L.*, Smith, A.E.* and Rosenberg, S.A. Surgery Branch, NCI-NIH, Bethesda, MD 20892. *Genzyme Corp., Framingham, MA 01701

The identification of the tumor antigens MART1 and gp100 by our group, which are specifically recognized by HLA-A2 restricted CD8+ CTLs derived from melanoma patients, has led us to develop antigen specific vaccines for the treatment of patients with metastatic melanoma. Replication defective recombinant adenoviruses Ad2CMV-MART1 and Ad2-CMV-gp100 have been successfully generated. Infection of non-antigen expressing HLA-A2+ cell lines A375 and MDA-231 with the vectors, resulted in recognition by the specific CTL as demonstrated by specific target cell lysis and release of cytokines. Sodium butyrate and TNFa can augment adenoviral mediated ene expression and increase cytotoxicity of specific CTLs. Though E3/19K protein was expressed at detectable level, significant reduction of surface MHC class I expression was observed in only 3 out of 10 tumor cell lines infected with either Ad2CMV-MART1 or Ad2CMV-gp100. The recombinant adenoviruses containing specific tumor antigen may be useful as vaccines for cancer therapy.

2928

The induction of cytotoxic T cells and tumor regression by soluble antigens in adjuvant formulation. K. Hariharan, G. Braslawsky, L. Berquist, A. Black, R. Barnett and N. Hanna. IDEC Pharmaceuticals, San Diego, CA 92121.

Class I-restricted cytotoxic T lymphocytes (CTL) induced by endocytic processing of tumor antigen presented via the MHC-1 molecules on the surface of the neoplastic cells play a major role in the immunity against cancer. In this study, we have demonstrated that CD8+ class I restricted CTLs can be elicited by injecting soluble ovalbumin (Ova) mixed in an Adjuvant Formulation (AF) which consists of microfluidized squalane, Tween 80 and Pluronic L121. To investigate the ability of this approach to generate anti-tumor immunity, C57BL/6 mice were immunized with soluble ovalbumin (Ova) in AF (Ova-AF) either 21 days before or 2 days after challenge with syngeneic, tumorigenic Ova expressing transfectoma (EG7; H-2^b, Ia$^-$). Significant inhibition of tumor growth was observed in mice immunized with Ova-AF, but not with Ova in Alum or Ova alone. Depletion of CD8$^+$ cells completely abrogated the AF-induced tumor protection, indicating that CD8$^+$ T cells were the major effector cells mediating tumor protection. This data indicates that AF, when combined with soluble protein antigens, provide an effective adjuvant for antigen specific class I restricted CTL response with potential utility in cancer therapeutic vaccines. Ongoing studies on tumor associated antigens incorporated into AF for the induction of tumor specific cell mediated immunity will be discussed.

XP-002120952

#2431 Induction of delayed-type hypersensitivity (DTH) to ovarian can... cells after treatment with an autologous (AUT), hapten-modified vacc...

Berd, D., Carlson, J., Bloome, E., Medley, W., and Dunton, C. *Thomas Jeffer... University, Philadelphia, PA 19107.*

Treatment of melanoma patients with a vaccine consisting of AUT tumor c... (TC) modified with the hapten, dinitrophenyl (DNP), induces DTH to DNP-modifi... TC (DNP-TC) in >95% of patients and to *unmodified* TC in 40–50%. The la... response was a significant predictor of survival in a post-surgical adjuvant stu... in stage III melanoma. To determine whether these results could be extended ... other tumors, we are conducting a study in patients with *stage III ovarian canc...* Tumor tissue was obtained during surgical debulking, and TC were dissocia... and cryopreserved. After 6 cycles of chemotherapy (taxol + cisplatin or carb... platin), patients received 6 weekly doses of DNP-vaccine preceded by a sing... dose of cyclophosphamide (300 mg/M^2). DTH was tested before treatment an... again 2 weeks after the last vaccine. Of 6 patients treated so far, all hav... developed positive DTH (≥ 5mm induration) to DNP-TC (median=16m... range=7–28mm). Unexpectedly all 6 developed DTH to mechanically-dissociate... *unmodified* TC as well (median=8mm, range=5–17mm). Thus, AUT DNP-vaccin... induces a T cell response in ovarian cancer that is at least equal to that obser... in melanoma. This result provides a rationale for a clinical efficacy trial.

Autologous Hapten-Modified Melanoma Vaccine as Postsurgical Adjuvant Treatment After Resection of Nodal Metastases

By David Berd, Henry C. Maguire, Jr, Lynn M. Schuchter, Ralph Hamilton, Walter W. Hauck, Takami Sato, and Michael J. Mastrangelo

Purpose: To determine whether treatment with an autologous whole-cell vaccine modified with the hapten dinitrophenyl (DNP vaccine) is an effective postsurgical adjuvant treatment for melanoma patients with clinically evident nodal metastases.

Patients and Methods: Eligible patients had regional nodal metastases that were large enough (≥ 3 cm diameter) to prepare vaccine. Following standard lymphadenectomy, patients were treated with DNP vaccine on a monthly or weekly schedule.

Results: Of 62 patients with metastasis in a single lymph node bed (stage III), 36 are alive after a median follow-up time of 55 months (range, 29 to 76); the projected 5-year relapse-free and overall survival rates are 45% and 58%, respectively. Of 15 patients with metastases in two nodal sites, five are alive with a median follow-up time of 73 months. An unexpected finding was the significantly better survival of older patients; the projected 5-year survival of patients greater than 50 versus ≤ 50 years was 71% and 47%, respectively ($P = .011$, log-rank test). The development of a positive delayed-type hypersensitivity (DTH) response to unmodified autologous melanoma cells was associated with significantly longer 5-year survival (71% v 49%; $P = .031$). Finally, the median survival time from date of first recurrence was significantly longer for patients whose subcutaneous recurrence exhibited an inflammatory response (> 19.4 v 5.9 months; $P < .001$).

Conclusion: Postsurgical adjuvant therapy with autologous DNP-modified vaccine appears to produce survival rates that are markedly higher than have been reported with surgery alone. Moreover, this approach has some intriguing immunobiologic features that might provide insights into the human tumor-host relationship.

J Clin Oncol 15:2359-2370.

PATIENTS WITH MELANOMA metastatic to regional lymph nodes have a relatively poor prognosis, especially when palpable lymph node masses are present.[1-6] Until recently no adjuvant therapy had shown a significant impact on relapse-free and overall survival. The string of negative results was broken by Kirkwood et al,[6] who reported an Eastern Cooperative Oncology Group (ECOG) study of high-dose interferon alfa-2b. Patients who received a 1-year course of interferon following surgery had significantly longer survival than those treated by surgery alone. This was most striking in patients with clinically evident lymph node metastases.

We have developed a novel approach to the treatment of human cancer: immunization with autologous tumor cells modified by the hapten, dinitrophenyl (DNP). Administration of DNP-modified melanoma vaccine has resulted in tumor regression in several patients with measurable metastases.[7] However, like other immunotherapies, the effectiveness is limited by tumor burden, possibly due to the production of immunosuppressive factors, such as interleukin-10, at the tumor site.[8] Therefore, it seemed reasonable to test the DNP vaccine in a setting in which the tumor burden is much lower. Patients with bulky but resectable regional lymph node metastases constitute an ideal group, since the metastatic masses provide a source of cells for preparing vaccine, but the postsurgical tumor burden is below the level of clinical detection. The preliminary results, which have been reported,[9] suggested an unexpectedly long relapse-free and overall survival in patients treated with DNP vaccine after lymphadenectomy. We now present a complete report of those trials, which confirms the preliminary results and provides some insights into the immunobiology of this treatment.

PATIENTS AND METHODS

Patients

The clinical characteristics of the study subjects are listed in Table 1. Sixty-two patients had American Joint Cancer Committee (AJCC) stage III melanoma (CS2, PS2) with at least one clinically evident lymph node metastasis that was at least 3 cm in diameter; the largest nodal metastases were 8 cm in diameter. Six of these patients had in-transit metastases as well. A group of 15 patients who had palpable masses in 2 lymph node sites was analyzed separately. These patients are considered to constitute a much worse prognostic group and, in fact, are sometimes classified as stage IV.[5] All patients underwent standard lymphadenectomy, including, when necessary, exci-

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Table 1. Patient-Related Variables

Variable	One Nodal Site (stage III)	Two Nodal Sites (stage IV)
Total patients	62	15
Sex		
Male	36	6
Female	26	9
Age, years		
Median	49	55
Range	21-83	30-72
Nodal site		
Axillary	36	—
Inguinal	19	—
Neck	5	—
Axillary + supraclavicular	—	4
Inguinal + pelvic	—	6
Bilateral axillary	—	3
Bilateral neck	—	2
No. of positive nodes		
1	31	—
2 or 3	20	3
≥ 4	11	12
In-transit metastases	6	0
Primary site		
Extremity	23	5
Trunk	31	8
Head & neck	4	2
Acrolentiginous	2	—
Unknown	2	—
Thickness of primary (mm)		
≤ 1.5	20	5
1.5-3.0	17	5
> 3.0	16	3
NA or unknown	9	2
Time to nodal metastasis		
< 3 months	10	3
3-12 months	18	6
1-3 years	17	1
> 3 years	15	5
HLA type		
$A2^+A3^-$	15	ND
$A3^+A2^-$	13	ND
$A2^+A3^+$	6	ND
$A2^-A3^-$	28	ND

Abbreviations: NA, not available; ND, not done.

sion of in-transit metastases. Computed tomography (CT) of the thorax, abdomen, and pelvis was performed in the perioperative period, and only patients without definite evidence of metastasis were eligible for DNP vaccine treatment. In all cases, the vaccine program was begun within 30 days of lymphadenectomy and the median time from lymphadenectomy was 21 days.

Vaccine Preparation

Tumor masses were obtained within 4 hours of surgery and were processed as previously described.[10] In brief, cells were extracted by enzymatic dissociation with collagenase and DNAse, aliquoted, frozen in a controlled-rate freezer, and stored in liquid nitrogen in a medium that contained 1% human albumin and 10% dimethylsulfoxide (DMSO) until needed. On the day that a patient was to be treated, the cells were thawed, washed to remove DMSO and irradiated to .25 Gy. Then, they were washed again and suspended in Hanks balanced salt solution without phenol red. Modification of melanoma cells with DNP was performed by the method of Miller and Claman.[11] This involves a 30-minute incubation of tumor cells with dinitrofluorobenzene (DNFB) under sterile conditions, followed by washing with sterile saline.

Each vaccine consisted of 5 to 25 × 10^6 live tumor cells (by trypan blue exclusion) suspended in 0.2 mL Hanks solution; there were variable numbers of residual lymph node lymphocytes in all specimens. After mixing with bacille Calmette-Guérin (BCG) (see later), the suspension was injected intradermally into three contiguous sites. The actual doses of vaccine administered are listed in Table 2.

Study Design

The studies were approved by the Institutional Review Board of Thomas Jefferson University and informed consent was obtained

Table 2. Treatment-Related Variables

Variable	Stage III Schedule A	Stage III Schedule B	Stage IV Schedules A + B
No. of patients	36	26	15
Mean dose per vaccine × 10^6 cells (mm)			
Median	13	10	10
Range	5-25	2-25	3-17
No. of vaccine doses administered (mm)			
Median	8	12	8
Range	2-8	6-12	1-12
Peak DTH to unmodified melanoma cells (mm)			
Median	5	2	4
Range	0-22*	0-6*	0-11
% ≥ 5 mm	51	8	21
% ≥ 10 mm	9	0	7
Peak DTH to DNP-modified melanoma cells (mm)			
Median	20	20	35
Range	7-55	9-70	7-60
% ≥ 5 mm	100	100	100
% ≥ 10 mm	85	96	90
Peak DTH to DNP-modified lymphocytes (mm)			
Median	16	16	15
Range	5-40	5-35	4-40
% ≥ 5 mm	100	100	93
% ≥ 10 mm	75	69	64
Peak DTH to PPD (mm)			
Median	25	23	21
Range	12-60	10-35	13-30
% ≥ 10 mm	100	100	100
% ≥ 20 mm	71	81	50

*Difference between stage 3, schedule A and stage 3, schedule B $P = .003$, Mann-Whitney U test.

Table 3. Schedules of DNP Vaccine Treatment

Schedules A and B	
Day −17	Cyclophosphamide
Day −14	DNFB sensitization
Day 13	DNFB sensitization
Day 0	Cyclophosphamide
Schedule A	
Day 3	DNP vaccine + BCG
Day 28	Cyclophosphamide
Day 31	DNP vaccine + BCG
Day 59	DNP vaccine + BCG
Day 87	DNP vaccine + BCG
Day 115	DNP vaccine + BCG
Day 143	DNP vaccine + BCG
Day 171	DNP vaccine + BCG
Day 199	DNP vaccine + BCG
Schedule B	
Day 3	DNP vaccine + BCG
Day 10	DNP vaccine
Day 17	DNP vaccine
Day 24	Unconjugated vaccine + BCG
Day 31	Unconjugated vaccine
Day 38	Unconjugated vaccine
Day 70	Cyclophosphamide
Day 73	DNP vaccine + BCG
Day 80	DNP vaccine
Day 87	DNP vaccine
Day 94	Unconjugated vaccine + BCG
Day 101	Unconjugated vaccine
Day 108	Unconjugated vaccine

from all patients. Two vaccine schedules were tested (Table 3). Schedule A was used for patients accrued from October 1989 to March 1993; schedule B was used for patients accrued from April 1993 to March 1994. For both schedules, patients were initially sensitized with DNFB by topical application of a 1% solution in acetone -corn oil on 2 consecutive days in the same site on the ventral upper arm; cyclophosphamide 300 mg/m^2 by rapid intravenous (IV) infusion was given 3 days before DNFB application.[12] In schedule A, DNP vaccine mixed with BCG (Tice; Organon Teknika Corp, Durham, NC) was administered every 4 weeks for a total of eight doses; cyclophosphamide 300 mg/m^2 was administered 3 days before the first and second doses. All vaccine injections were given in the same site on a limb (usually the upper dorsal arm) that had not been subjected to a lymph node dissection. In schedule B, vaccine was administered weekly for 6 weeks; after a 4-week reevaluation period, vaccine was again administered weekly for 6 weeks. The first three vaccines of each course were DNP-modified and the last three were unmodified. BCG was admixed only with the first and fourth vaccine of each course. All of the DNP vaccine injections were given into one area, and all of the unmodified vaccine injections were given into a second area. Cyclophosphamide 300 mg/m^2 was administered 3 days before the start of each vaccine course.

For both schedules, the dose of BCG was progressively attenuated to produce a local reaction that consisted of an inflammatory papule without ulceration. The attenuation schedule was as follows: (1) 0.1 mL of a 1:10 dilution (1 to 8 × 10^6 colony-forming units [CFU]); (2) 0.1 mL of a 1:100 dilution (1 to 8 × 10^5 CFU); and (3) 0.1 mL of a 1:1,000 dilution (1 to 8 × 10^4 CFU). Because of the progressive development of cell-mediated immunity, most patients were receiving the lowest BCG dose by the fourth BCG injection.

Following the completion of vaccine treatments, patients were evaluated at the Thomas Jefferson University Hospital every 2 months for 2 years, every 3 months for the third year, and every 6 months thereafter. Laboratory evaluations were as follows: complete blood cell count, liver function tests, and chest x-ray every 2 months; and CT of the chest, abdomen, and pelvis every 6 months. No patients were lost to follow-up evaluation.

Skin Testing

Patients were tested for delayed-type hypersensitivity (DTH) by a standard method that we have previously described.[10] Cryopreserved melanoma cell suspensions and peripheral-blood lymphocytes (PBL) were thawed, washed, and irradiated (.25 Gy). Only mechanically dissociated melanoma cell suspensions were used in view of our previous observation that patients immunized with enzyme-dissociated melanoma cells develop strong DTH to collagenase and DNAse.[13] DNP modified melanoma cells and PBL were prepared as described earlier. A total of 1 × 10^6 melanoma cells and 3 × 10^6 PBL, each either DNP-modified or unmodified, were suspended in Hanks balanced salt solution without serum, phenol red, or antibiotics and injected intradermally into the ventral forearm. The mean diameter of induration was measured after 48 hours. Patients were also skin-tested with intermediate-strength purified protein derivative (PPD; 5 tuberculin units [TU]). DTH testing was performed before DNFB sensitization and then every 2 months during the time of administration of DNP vaccine. Table 2 summarizes the results of DTH testing. No patients exhibited DTH responses to autologous, unmodified PBL, either before or after treatment, which excludes the possibility of spurious responses to the cryopreservation medium or its components.

Human Leukocyte Antigen Testing

Initially, class I typing was performed by the Tissue Typing Laboratory of Thomas Jefferson University Hospital in 20 patients, using standard serologic methods. After preliminary analysis suggested that patients who expressed human leukocyte antigen (HLA)-A3 had a shorter survival than patients with other phenotypes, all of the stage III patients were tested for expression of A3 and A2 by using monoclonal antibodies (American Type Culture Collection, Rockville, MD: A2, HB82; A3, HB122) and flow cytometry. In all cases, the two methods yielded identical results.

Statistics

Survival was plotted by the Kaplan-Meier method and the difference between survival curves was determined by the log-rank test of Mantel. The effect of prognostic variables on survival was determined by proportional hazards regression (Cox).

RESULTS

Stage III: Relapse Free and Overall Survival

The relapse-free survival of the 62 patients with clinical stage III melanoma treated postlymphadenectomy with DNP-modified vaccine is shown in Fig 1. The median relapse-free survival duration is between 24 and 37 months and the projected 5-year relapse-free survival rate is 45%. All patients have been monitored for at least 29 months and the median follow-up time is 55 months. Of six patients who had in-transit metastases, five are alive and melanoma-free at 44, 45, 60, 60, and 68 months—four continuously relapse-free and one long-term relapse-

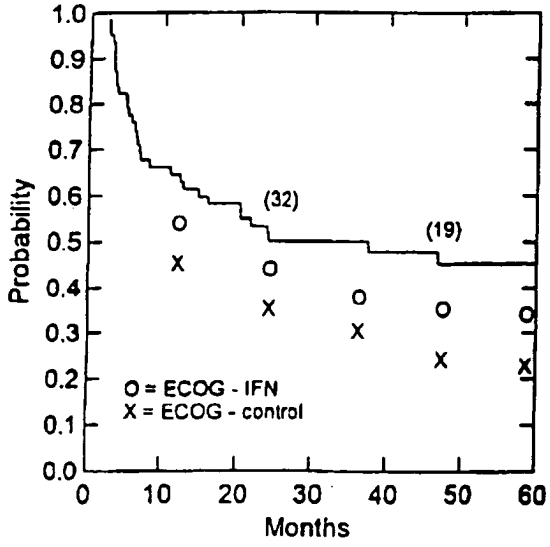

Fig 1. Relapse-free survival of patients with stage III melanoma treated with vaccine. (X, O), relapse-free survival at selected time points of clinical stage III patients treated in the ECOG adjuvant study; (O), surgery alone; (X), surgery + high-dose interferon. (Data from Kirkwood et al.[4])

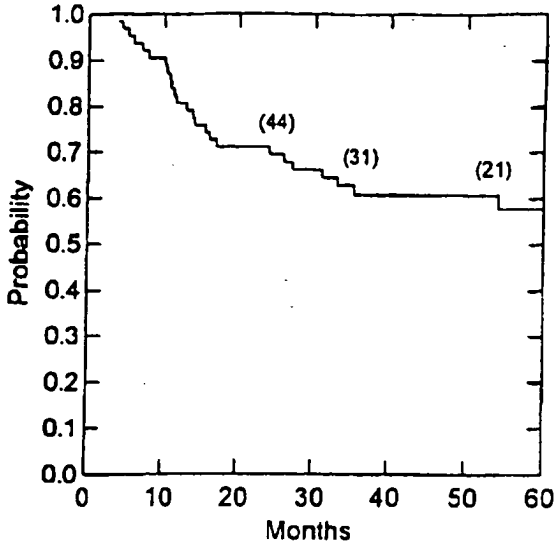

Fig 2. Overall survival of patients with stage III melanoma treated with vaccine.

free after resection of a single recurrence. To provide a comparison with standard therapy, Fig 1 has been annotated to show relapse-free survival at selected points for patients in the ECOG study group who received high-dose interferon alfa-2b and for the untreated (surgery-only) controls.[6] Figure 2 shows the overall survival of these 62 patients. The median overall survival time is more than 62 months and the projected 5-year survival rate is 58%. Since the report by Kirkwood et al[6] did not provide overall survival data for this group of patients, their data could not be shown on this graph.

Univariate Analysis of Prognostic Variables

We performed a univariate analysis of a series of patient-related and treatment-related variables to determine their impact on relapse-free and overall survival of these 62 stage III patients.

Of the patient-related variables examined, age, number of positive nodes, and HLA type had a significant ($P < .05$) impact on survival (Tables 1 and 4). The importance of the extent of nodal involvement is shown in the Kaplan-Meier plot in Fig 3. Patients with a large palpable metastatic mass but no other microscopic nodal involvement had a project 5-year survival rate of 72% versus 34% in patients with four or more positive nodes. The effect of age is shown in Fig 4; older patients (> 50 years) fared significantly better than younger patients. This finding was unexpected, since the ECOG interferon study reported the opposite result,[6] and in other studies, age has been a neutral factor.[2] Even more surprising was the effect of HLA class I phenotype: the 13 patients who were $A3^+A2^-$ had a median survival time of only 11 months, compared with more than 30 months for patients who expressed A2 (with or without A3) or who did not express either A2 or A3. Sex and site or thickness of the primary melanoma were not statistically significant predictors of survival.

It is of particular interest that the interval from treatment of the primary cutaneous melanoma to the time of development of a clinically apparent nodal metastasis was not a significant variable for either relapse-free or overall survival. Patients with a clinically evident nodal metastasis at the time of presentation of the primary melanoma (ie, time to nodal metastasis = 0) have been reported to have a particularly poor prognosis (5-year relapse-free survival rate, < 10%[6]). Of eight such patients in our trial, four are alive at 37, 44, 54, and 67 months, respectively.

Of the treatment-related variables, the development of a positive (≥ 5 mm) DTH response to unmodified autologous melanoma cells was associated with significantly longer 5-year survival (71% *v.* 49%) (Tables 2 and 5; Fig 5). In contrast, the other DTH responses that developed following administration of DNP vaccine (DNP-modified autologous melanoma cells or lymphocytes, PPD) were not predictive, except that the development of a large PPD response was associated with shorter survival, which

Table 4. Univariate Association of Patient-Related Variables With Relapse-Free and Total Survival—Stage III

Factor	No. of Patients	Median Time to Recurrence (months)	P	Median Time to Death (months)	P
Sex			.257		.431
Male	36	22		54	
Female	26	> 16		> 35	
Age, years			.002		.011
≤ 50	35	10		32	
> 50	27	> 47		> 54	
No. of positive nodes			.003		.049
Mass only	31	> 61		> 62	
Mass and 1-2 micrometastes	20	15		35	
Mass and ≥ 3 micrometases	1*	5		13	
Primary site			.094		.191
Extremity	23	47		> 54	
Trunk	31	17		36	
Other	8	> 20		> 33	
Thickness of primary (mm)			.771		.569
≤ 2.5	31	37		56	
> 2.5	22	24		> 27	
NA or unknown	9	17		> 33	
Time to nodal metastasis, months			.296		.252
≤ 12	28	37		> 27	
> 12	32	20		54	
HLA type			.003		.001
$A2^+,A3^-$	15	22		> 33	
$A3^+,A2^-$	13	5		11	
$A2^+,A3^+$	6	> 35		> 35	
$A2^-,A3^-$	28	49		> 63	

*Log-rank test.

was of boarderline significance. Schedule of administration (A v B) and average vaccine dose were not statistically significant variables.

Multivariate Analysis of Prognostic Variables

A multivariate analysis was performed using a Cox model. Among patient-related variables (Table 6), more extensive nodal involvement was associated with a significantly higher hazards ratio for both relapse-free and overall survival. Age more than 50 years was associated with significantly lower hazards ratios (0.29 and 0.37 for relapse-free and overall survival, respectively). Therefore, the treatment-related variables were analyzed after adjusting for age and number of positive nodes. As shown in Table 7, the failure to develop DTH to unmodified autologous melanoma cells was associated with a hazards ratio of 2.54 for overall survival, but this was of boarderline statistical significance (P = .080). Because of the possibility that patients with more extensive nodal involvement also might be less likely to develop a cell-mediated immune response to melanoma antigens, we modeled DTH to unmodified autologous melanoma cells after adjustment for age only. The hazards ratios for both relapse-free and overall survival increased and were statistically significant (P = .029 and .036, respectively).

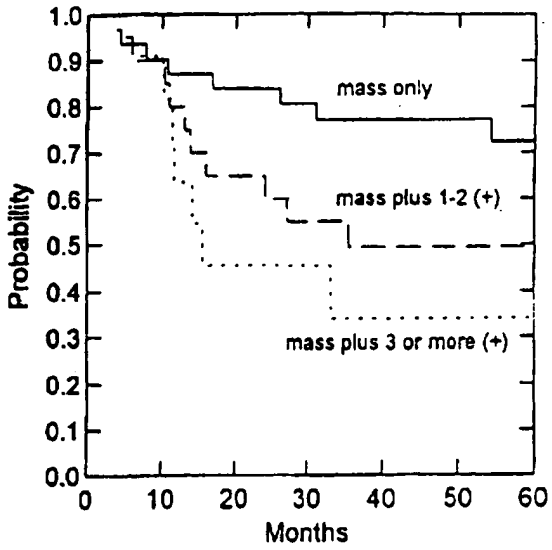

Fig 3. Overall survival of patients with stage III melanoma treated with vaccine stratified by degree of lymph node involvement. P = .049, log-rank test, 2-tailed.

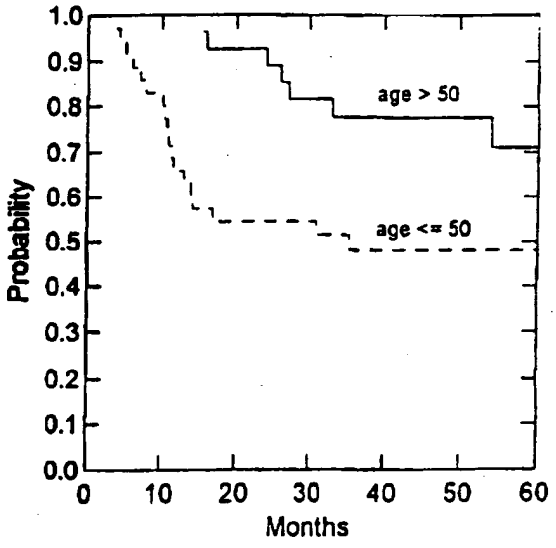

Fig 4. Overall survival of patients with stage III melanoma treated with vaccine stratified by age. P = .011, log-rank test, 2-tailed.

Table 5. Univariate Association of Treatment-Related Variables With Relapse-Free and Total Survival—Stage III

Factor	No. of Patients	Median Time to Recurrence (months)	P*	Median Time to Death (months)	P
Schedule			.265		.512
A	36	46		> 63	
B	26	13		> 27	
Mean vaccine dose			.922		.850
≤ 10 × 10⁶ cells	29	23		54	
> 10 × 10⁶ cells	33	25		> 63	
DTH to unmodified melanoma cells (mm)			.028		.031
≥ 5	19	> 46		> 54	
< 5	38	13		33	
DTH to DNP-modified melanoma cells (mm)			.502		.400
≥ 20	20	12		> 16	
< 20	16	6		24	
DTH to DNP-modified lymphocytes (mm)			.298		.331
≥ 15	31	46		> 62	
< 15	31	16		> 27	
DTH to PPD (mm)			630		.100
≥ 25	28	24		42	
< 25	28	37		> 35	

*Log-rank test.

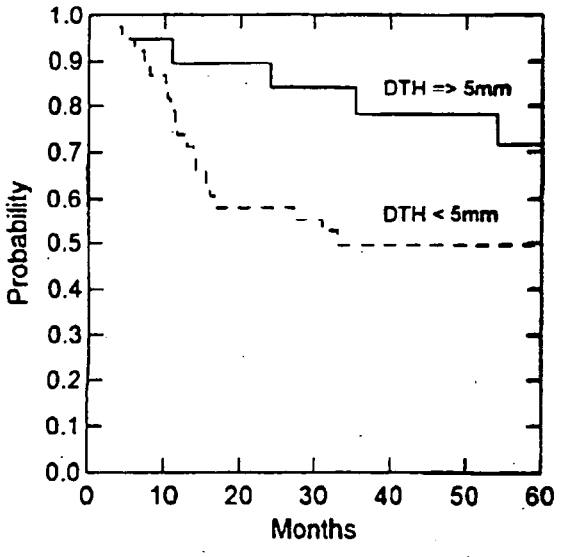

Fig 5. Overall survival of patients with stage III melanoma treated with vaccine stratified by peak DTH to unmodified autologous melanoma cells. P = .031, log-rank test, 2-tailed.

The trend toward lower overall survival in patients with large DTH responses to PPD, noted in the univariate analysis, was also evident in the Cox model. This effect disappeared when that parameter was modeled after adjustment for age only, which suggests that it was a reflection of the higher hazards ratios of younger subjects.

It was not possible to generate a Cox model that included HLA type. However, log-rank tests of HLA type stratified by age and number of positive nodes confirmed the results of the univariate analysis of this parameter: HLA type remained a significant predictor of relapse-free and overall survival (P = .016 and .014, respectively).

Patients With Metastases to Two Lymph Node Sites

We treated 15 patients with autologous DNP vaccine after resection of bulky metastases to two nodal sites, generally classified as stage IV.[2] Five of 15 are alive and melanoma-free at 32, 39, 73, 76, and 81 months—three continuously relapse-free and two long-term relapse-free after resection of a single recurrence. The sites of nodal metastases in these five patients were as follows: inguinal plus pelvic, n = 3; bilateral axillary, n = 1; and axillary plus neck, n = 1.

Sites and Treatment of Relapses

A total of 46 patients developed recurrence of melanoma following DNP vaccine treatment. The anatomic sites of first recurrence were as follows: soft tissue, n = 26; lung, n = 5; liver, n = 5; bone, n = 4; brain, n = 5, and intraabdominal, n = 1. This distribution of sites of recurrence is similar to that reported in a large retrospective study.[14] It is noteworthy that brain metastases were uncommon, since the brain is considered by some investigators to be an immunologically privileged site that would not be protected by the development of active tumor immunity.[15]

The management of patients following relapse was not specified in the vaccine protocol; instead, patients received what was considered to be standard of care by their physicians. Initial postrelapse treatments were as follows: chemotherapy, 24 patients; surgery (usually complete excision of one or two soft tissue metastases), 18 patients, six of whom subsequently received chemotherapy; and no treatment (because of patient refusal or rapidly progressive metastases), four patients. Of 30 patients who received combination chemotherapy,[16] there were eight partial responses with a median duration of 3 months and no complete responses. Chemotherapy did not appear to have an impact on postrelapse survival. Of 18 patients who underwent surgery, eight were treated with a second course of DNP vaccine, made by excising

Table 6. Multivariate Analysis (Cox model)—Stage III—Known Prognostic Factors

Factor	Relapse-Free Survival			Overall Survival		
	Hazards Ratio*	95% CI	P	Hazards Ratio*	95% CI	P
Sex, female	0.90	0.37-2.21	.822	0.84	0.32-2.25	.733
Age > 50 years	**0.29**	**0.11-0.75**	**.008**	**0.37**	0.13-1.04	**.053**
No. of positive nodes (v mass only)			**.001**			**.030**
Mass and 1-2 micrometases	**2.43**	1.06-5.58		**2.48**	0.99-6.23	
Mass and ≥ 3 micrometases	**6.54**	2.25-19.02		**3.80**	1.22-11.84	
Primary site (v extremity)			.565			.174
Trunk	1.07	0.45-2.53		†		
Other	0.36	0.04-3.12				
Thickness of primary (v ≤ 2.5 mm)			.876			.592
> 2.5	0.90	0.39-2.10		0.59	0.22-1.62	
Unknown	1.21	0.49-2.75		0.77	0.20-3.07	
Time to nodal metastasis > 12 months	1.16	0.49-2.75	.730	1.23	0.50-3.01	.647

Abbreviation: CI, confidence interval.

*Hazards ratios with $P < .100$ are highlighted in boldface.

†Results are for Cox analyses, including all variables listed. The exception is that Cox regression could not fit primary site for analysis of overall survival. Therefore, the P value is adjusted only for age and no. of positive nodes. The overall survival results for the other factors are not adjusted for primary site.

and processing the recurrent tumor tissue. Of this group, six have died and two are surviving relapse-free at 25 and 26 months postrecurrence, respectively.

Histology of Recurrent Tumors

Fourteen patients had skin (subcutaneous or dermal) metastases as the initial site of recurrence. Seven of these tumors exhibited inflammatory responses that were histologically similar to those that we have reported in patients who received DNP vaccine for treatment of measurable metastases.[7,17] A representative histology is shown in Fig 6. We calculated the time from first recurrence of melanoma to death and determined whether tumor inflammation was a significant prognostic variable. The median survival times were as follows: inflammation in recurrent tumors, greater than 19.4 months; and no inflammation, 5.9 months ($P < .001$, log-rank test) (Fig 7). A multivariate analysis of prognostic variables for postrelapse survival for this group showed that tumor inflammation was the only statistically significant predictor of longer survival. For example, whether subcutaneous metastases were completely resected was not a significant factor in uinvariate or multivariate analyses.

Toxicity

Systemic toxicity was almost entirely attributable to cyclophosphamide, and, as expected with the low dose, it was mild. Approximately one third of patients reported short-lived nausea or vomiting, which in most cases was grade 1 or 2. One patient treated on schedule B developed generalized urticaria 15 minutes after injection of her fourth dose of DNP vaccine; this abated spontaneously and was not associated with other symptoms of hypersensitivity. The vaccine was discontinued and this patient has remained tumor-free for 5 years. Otherwise, the toxicity of the DNP vaccine was limited to local reactions at

Table 7. Multivariate Analysis (Cox model)—Stage III—Treatment-Related Variables

Factor	Relapse-Free Survival			Overal Survival		
	Hazards Ratio*	95% CI	P	Hazards Ratio*	95% CI	P
Adjusted for age and no. of positive nodes						
Schedule B	1.67	0.80-3.47	.167	1.44	0.61-3.39	.398
Mean vaccine dose > 10 x 10⁶	1.26	0.60-2.65	.542	1.12	0.48-2.57	.797
DTH to unmodified melanoma cells < 5 mm	2.03	0.82-5.02	.119	2.54	0.87-7.42	.080
DTH to DNP-modified melanoma cells < 20 mm	0.74	0.31-1.76	.494	1.11	0.39-3.14	.846
DTH to DNP-modified lymphocytes < 15 mm	1.19	0.59-2.39	.626	1.01	0.44-2.30	.988
DTH to PPD < 25 mm	0.64	0.30-1.36	.245	0.40	0.16-1.01	.046
Adjusted for age only						
DTH to unmodified melanoma cells < 5 mm	2.49	1.07-5.82	.029	2.81	1.03-7.65	.036
DTH to PPD < 25 mm	0.94	0.46-1.92	.872	0.65	0.26-1.62	.352

*Hazards ratios with $P < .100$ are highlighted in boldface

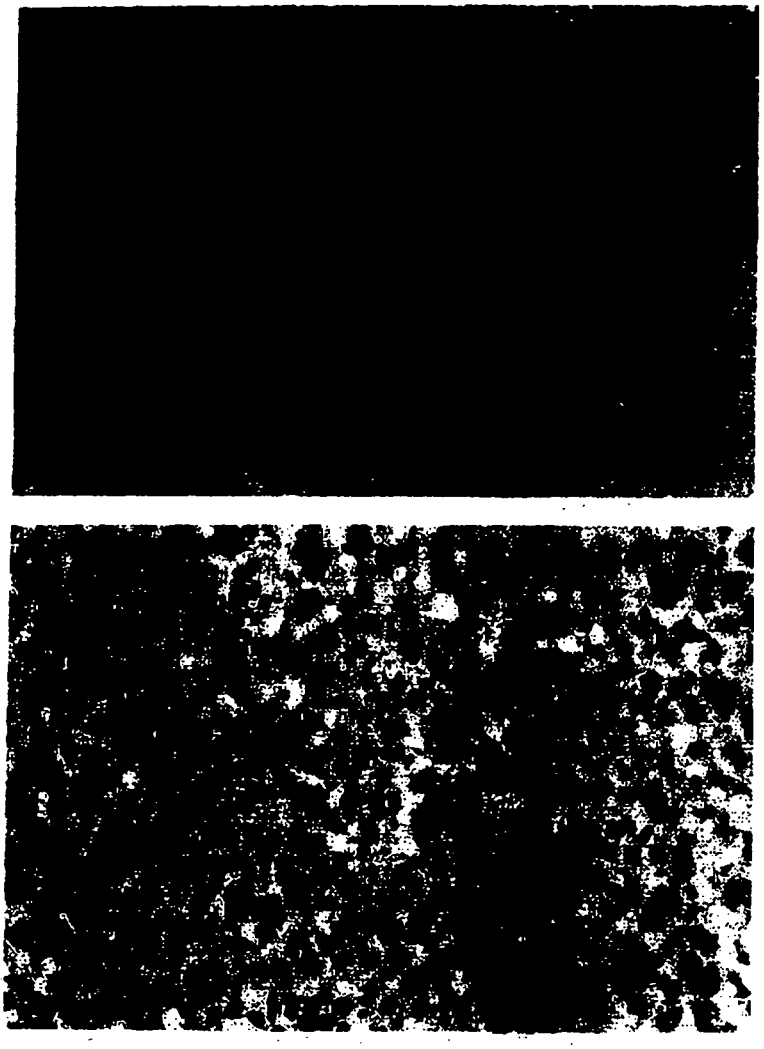

Fig 6. Histology of a subcutaneous metastasis as the first site of melanoma recurrence. Original magnifications: (A) 100×; (B) 400×. Note the dense infiltration of lymphocytes into the tumor tissue. Clinically, this lesion was a 5-mm diameter subcutaneous mass on the mid abdomen.

the vaccine sites. All patients developed pruritic papules that progressed to pustules, sometimes with small ulcerations; the intensity of the reactions was ameliorated by reducing the dose of BCG. No patients noted fever or chills following vaccine administration and no patient experienced a decrease in performance status. Finally, no systemic autoimmune phenomena (eg, arthritis or cutaneous vasculitis) were observed. Vitiligo was never seen.

DISCUSSION

We have developed a novel approach to the treatment of human cancer: immunization with autologous tumor cells modified by the hapten, DNP. The regimen incorporates the administration of low-dose cyclophosphamide before immunization because of its ability to augment cell-mediated immune responses.[10,18] In an initial report, we demonstrated that patients with metastatic melanoma administered a DNP-modified vaccine developed inflammatory responses in metastatic tumor masses.[17] Immunohistochemistry and flow cytometric analysis of biopsy specimens showed infiltration with lymphocytes, the majority of which were $CD8^+$, $HLA\text{-}DR^-$ T cells.[17,19] Polymerase chain reaction (PCR)-based analysis of these tissues indicates that the T cells produce interferon gamma.[20] Recently we, in collaboration with the group

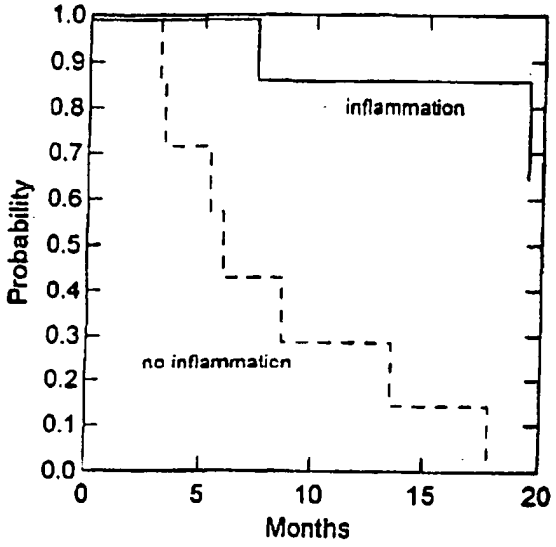

Fig 7. Effect of tumor inflammation on survival from first relapse. Upper curve (——), survival of 7 patients with first recurrence showed an inflammatory response. Lower curve (– – –), survival of 7 patients with first recurrence showed no evidence of an inflammatory response. $P < .001$, log-rank test, 2-tailed.

at the Istituto Nazionale in Milan, have reported that the infiltrating T cells represent expansion of a restricted set of T-cell receptor V-beta gene families, and that in two of two patients studied, the T-cell expansion was clonal.[21] These clones were not found in noninfiltrated metastases excised before administration of DNP vaccine. These observations suggest that immunization with DNP-modified autologous melanoma induces a T-cell response at the tumor site that is driven by yet-to-be-identified melanoma antigens.

The rationale for the use of a hapten-modified human tumor vaccine is well established. It is known that immunization of mice with TNP-modified lymphocytes results in the development of splenic T cells that exhibit secondary proliferative and cytotoxic responses to trinitrophenyl (TNP)-modified cells in vitro.[22] These responses are sometimes associated with low-grade, but reproducible, cross-reactivity with unmodified autologous targets/stimulators. This phenomenon has acquired clinical relevance through the work of Neurath et al,[23] who induced autoimmune colitis in mice by a single application of a hapten to the rectal mucosa. Moreover, it is the basis of drug-induced autoimmune disease: drugs act as haptens, which combine with normal tissue protein-forming immunogenic complexes that are recognized by T cells.[24] Subsequently, autoimmune disease, eg, systemic lupus erythematosus, can develop and continue even after withdrawal of the offending drug.[25,26]

The existence of T cells generated by hapten-modified cells that are reactive to both modified and unmodified cells has recently been directly demonstrated. Ortmann et al[27] have shown that class I major histocompatability complex (MHC)-restricted T-cell clones generated from mice immunized with TNP-modified syngeneic lymphocytes respond to MHC-associated, TNP-modified self-peptides. Furthermore, some TNP-reactive clones respond to certain MHC-binding, unmodified peptides as well.[28] A similar observation has been made with murine T-cell hybridomas responsive to hen egg lysozyme (HEL) modified with the hapten phosphorylcholine (PC).[29] The immunochemical basis of this phenomenon remains speculative, but several hypotheses are being tested. For example, Martin et al[28] have explained their results by hypothesizing the existence of autoreactive T cells that escape thymic selection because of low affinity for self-peptides. Hapten modification of such peptides may convert subdominant peptide epitopes into dominant determinants and thereby activate those T cells.

The current results suggest that administration of autologous DNP vaccine may be an effective postsurgical adjuvant treatment in patients with bulky, regional lymph node metastases. The 5-year relapse-free survival (45%) and overall survival (58%) rates in our series appear to be considerably higher than survival rates achieved with lymphadenectomy alone. Slingluff et al,[4] in a retrospective study of 4,682 patients, found that the "incidence of distant metastases closely mirrored the incidence of regional metastases." This generalization seems to be supported by data from a number of other published reports. For example, Balch et al[1] reported a 5-year survival rate of 24% for patients with clinically detectable nodal metastases. Karakousis et al[2] observed that of 111 patients with palpable positive nodes, only 18% survived 5 years. In the Sloan-Kettering series of 1,019 patients reported by Coit et al,[3] the 5-year survival rate of patients with palpable nodes more than 3 cm in diameter in one nodal site was 22%; when two nodal sites had palpable metastases, the 5-year survival rate decreased to 8%. Finally, in a study reported by Retsas et al,[5] the 5-year survival rate of 169 patients with clinically detectable nodal metastases treated with surgery alone was 28% and the relapse-free survival rate was approximately 10%.

Similar survival statistics have been reported in the ECOG study of interferon alfa-2b as postsurgical adjuvant therapy of melanoma.[6] This landmark study showed that administration of high-dose interferon significantly increased relapse-free and overall survival. The 5-year re-

lapse-free survival rate of surgical controls with clinically evident nodal metastases was approximately 23%, which was increased to approximately 33% with interferon treatment.[6] In our study, administration of DNP vaccine resulted in a relapse-free survival that is twice that of the ECOG control group and somewhat higher than that of the high-dose interferon group.

Although this comparison is weakened by the fact that it is historical rather than prospective, it is strengthened by the inclusion in our trial of patients who would be expected to have a particularly poor prognosis. Eight patients presented with palpable lymph node metastases at the same time that their primary melanoma was diagnosed, an occurrence that has been associated with a 5-year relapse-free survival rate of less than 10%.[6] Six patients had in-transit metastases, as well as clinically evident lymph node metastases. Finally, we did not exclude patients with extranodal extension of melanoma, because tumor processing precluded microscopic evaluation of this feature. In fact, some of the tumors that we processed were quite large (> 5 cm) and may have consisted of smaller, matted nodes—evidence of invasion of the lymph node capsule.

Analysis of the effect of patient-related and treatment-related variables on survival after administration of DNP vaccine disclosed several associations that may be important in understanding the immunology of this therapeutic approach. One striking finding was the superior outcome of older patients. In both univariate and multivariate analyses, patients greater than 50 years of age had significantly longer relapse-free and overall survival times. This is opposite to the age effect noted in the ECOG interferon study, in which older patients had a significantly worse outcome. The reason for our result is, of course, speculative. A provocative but testable hypothesis is that the increased tendency toward autoimmunity associated with aging makes it easier to break tolerance to tumor antigens by active immunization.[40] A peculiar finding was the poor outcome in patients with the HLA phenotype, $A3^+,A2^-$. Since we did not perform complete HLA analysis in our patients, this observation could be dismissed as artifactual. However, before doing so, one should consider that two varieties of putative autoimmune disease occur at a much lower frequency in people who express HLA-A3.[41,42] Thus it is possible that this phenotype is associated with diminished immune responsiveness in certain situations.

Finally, we found that patients who developed DTH to autologous, unmodified melanoma cells had significantly longer survival following treatment with DNP vaccine. This observation strengthens our claim that DNP vaccine works by inducing an antimelanoma immune response. Also, it provides an important immunologic parameter by which the effectiveness of new vaccine approaches can be measured. It is important to note that the magnitude of DTH responses to DNP-modified melanoma cells was uniformly high and was not predictive of clinical outcome. We believe that the development of cell-mediated immunity to hapten-modified cells is necessary, but not sufficient for the generation of an antitumor response.[28]

Little is known about the importance of dosage schedule for the effectiveness of human tumor vaccines. Our original dosage schedule (schedule A) was based on work previously performed with a less successful, nonhaptenized autologous melanoma vaccine.[10] After analyzing the immunologic results of schedule A, we made a strategic decision to test a second dosage schedule (B) that was more intensive and might be expected to induce stronger DTH responses more rapidly. Moreover, in a pilot study of schedule B in melanoma patients with measurable metastases, we observed a striking case of 95% regression of multiple lung metastases (unpublished observation). The results of the current study do not support our hypothesis, because (1) the clinical effects of schedules A and B (ie, relapse-free and overall survival) were not significantly different; and (2) DTH to unmodified autologous melanoma cells was actually greater with schedule A. Thus, at this writing, we cannot recommend one schedule over the other, and additional studies of the immunologic and clinical effects of DNP vaccine dosage and frequency of administration are in progress.

The finding of tumor inflammatory responses in the initial sites of relapse in seven of 14 patients in whom this could be evaluated (ie, skin metastases) was unexpected. To our knowledge, this phenomenon has not been previously reported in either surgical series or in patients receiving adjuvant therapies. The histology of these responses closely resembles the tumor inflammatory responses induced by DNP vaccine in patients with clinically evident metastases,[7,17] and suggests an incipient immune reaction against the recurrent tumor. The observation that inflammation of recurrent tumors was associated with prolonged survival indicates that the response was clinically meaningful.

This is the largest published trial of an autologous tumor vaccine as a postsurgical adjuvant therapy. Although the results must be interpreted with caution in the absence of a concomitant control group, patients who received DNP vaccine appear to have markedly higher relapse-free and overall survival rates than have been reported with surgery alone. Moreover, the survival percentages are comparable to, and may be higher than, those

of patients treated with high-dose interferon, and were achieved with minimal toxicity. Finally, DNP vaccine has some intriguing immunobiologic features—increased effectiveness in older subjects, possible HLA association, induction of tumor DTH responses, and inflammation in recurrent tumor sites—that have not been reported in other human tumor vaccine trials.[33,34]

There are practical considerations for the large-scale testing and application of an autologous melanoma vaccine. The vaccine requires the availability of at least 5 g of tumor tissue (a 2- to 3-cm diameter mass) and a laboratory that is capable of processing it. We have demonstrated the feasibility of acquiring and processing tumor specimens and preparing and administering haptenized vaccines for the treatment of 50 to 75 patients per year. Although the procedures are relatively simple, upscaling them to provide treatment for hundreds or thousands of patients at multiple sites would be a challenging task that would require the resources of a biotechnology company. However, we believe that, given our promising clinical results, it should now be possible to conduct an adequately powered phase III clinical trial to compare DNP vaccine with an established treatment. Plans for such a trial are in progress.

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Dinitrophenyl-Modified Autologous Melanoma Vaccine Induces a T Cell Response to Hapten-Modified, Melanoma Peptides

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Active specific immunotherapy with dinitrophenyl (DNP)-modified autologous melanoma vaccine elicits inflammatory responses in metastatic tumor sites. Postsurgical adjuvant immunotherapy with this vaccine prolongs survival in stage III melanoma patients. We have reported that, after administration of DNP-modified melanoma vaccine, T cell responses to DNP-modified autologous tumor cells are demonstrable *in vivo* and *in vitro*. These responses are hapten specific and MHC restricted. To elucidate this phenomenon, we investigated the immune response to DNP-modified peptides eluted from autologous cells. Short peptides were extracted from DNP-modified and unmodified autologous melanoma cells by an acid elution technique and HPLC fractionation. Peptides were also extracted from DNP-modified and unmodified, EB virus-transformed, autologous B lymphoblasts. These various peptide fractions were loaded onto autologous B lymphoblasts and tested for ability to elicit a response by a DNP-specific T cell line as measured by IFN-γ production. Unexpectedly, stimulatory activity of peptides from DNP-modified melanoma cells was confined to a single HPLC fraction. Spectrometric analysis of this fraction confirmed modification of peptides with DNP. A weaker T cell response was observed to a single HPLC fraction of DNP-modified peptides from the patient's B lymphoblasts. No T cell response was elicited by corresponding fractions of peptides eluted from unmodified melanoma cells or B lymphoblasts. These findings demonstrate the human T cell response to DNP-modified autologous melanoma cells is mediated by hapten-modified, MHC-associated peptides. Further investigation of these peptides could lead to a new strategy for peptide-based cancer immunotherapy.

INTRODUCTION

Successful immunotherapy against human cancer requires the induction of a cell-mediated immune response to weakly immunogenic tumor-associated antigens. Recent advances in technology have made it possible to identify tumor-related peptides associated with MHC molecules that elicit cytotoxic T cell responses (1–3), and clinical applications of peptide-based vaccine are now under investigation (4, 5).

Our group has been investigating a hapten-modified autologous melanoma cell vaccine (6). Melanoma cell suspensions obtained from metastatic masses are modified with a hapten, dinitrophenyl (DNP), and administered intradermally with BCG after pretreatment with low-dose cyclophosphamide. This treatment induces inflammatory responses in metastases (6) and also significantly increases disease-free survival and total survival in patients with bulky, resectable nodal metastases (7, 8). Immunohistochemical and flow cytometric analyses of postvaccine, inflamed metastases shows marked infiltration with $CD8^+$ lymphocytes (9), and some of these specimens contain m-RNA for IFN-γ (10). Furthermore, Sensi *et al.* recently reported the appearance of novel TCRVβ structures in metastatic melanomas in which inflammation was induced by DNP-modified vaccine (11). Patients who receive DNP-melanoma vaccine develop a strong delayed type hypersensitivity (DTH) response to DNP-modified autologous melanoma cells and, to a lesser extent, to DNP-modified autologous PBL (12). This is reflected *in vitro* by proliferation and cytokine production by PBL obtained from patients following vaccination, but not prior to vaccination. Previous studies of a $CD8^+$ T cell line derived from such PBL indicate that the response to DNP-modified autologous cells was class I MHC restricted. Furthermore, the T cell line responds to allogeneic DNP-modified stimulators that are matched at one or both HLA loci, but not to stimulators that are HLA mismatched (12).

In this study, we investigate the epitopes recognized by these DNP-specific T cells. We demonstrate that the T cells respond to small, DNP-modified peptides associated with the MHC. Unexpectedly, the stimula-

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tory activity appeared to be limited to a single HPLC peptide fraction.

MATERIALS AND METHODS

Cells

PBL were obtained from a patient who developed a strong DTH reaction to DNP-modified autologous melanoma cells following DNP vaccine administration. PBL were separated from blood by density gradient centrifugation, suspended in freezing medium, frozen in a control-rate freezer, and stored in liquid nitrogen until use (12).

A T cell line was established from these PBL by repeated stimulation with DNP-modified autologous melanoma cells (DNP-Mel) as descrived previously (12). In brief, PBL (2×10^6) were mixed with autologous DNP-modified melanoma cells (4×10^5) in 24-well flat-bottom plates in lymphocyte culture medium (RPMI 1640 supplemented with 10% human AB serum, 2 m*M* L-glutamate, 100 μg/ml /100 U/ml streptomycin/penicillin, 10 m*M* Hepes, 1% nonessential amino acids). After 5 days of culture, recombinant interleukin 2 (IL-2) (a gift of Chiron, Emeryville, CA) was added at 100 U/ml. Expanding T cell cultures were maintained in culture medium with IL-2 and were split as needed to maintain a concentration of approximately 2×10^6 cells in a 22-mm-diameter well. The cultures were restimulated with DNP-modified autologous tumor cells every 2 weeks. After 4 weeks of incubation, a T cell line that was specific for DNP-modified autologous melanoma cells was established. The T cell line used for peptide experiments consisted of 51% $CD4^+$ cells and 48% $CD8^+$ cells at the time of study as determined by flow cytometry. This T cell line responded to DNP-modified autologous melanoma cells by proliferation and IFN-γ production. IFN-γ production by the T cell line after stimulation with DNP-modified autologous melanoma cells was completely inhibited by an anti-MHC class I antibody but not inhibited by an anti-MHC class II antibody (inhibition of 91 and 12.5%, respectively).

B lymphoblastoid cells were obtained by EB virus transfection using standard techniques (12).

Melanoma cells were enzymatically extracted from metastatic masses from the same patient and cryopreserved by a previously described method (9). An autologous melanoma cell line was established from the melanoma cell suspension in our laboratory. This cell line is positive for melanoma-associated proteoglycan detected by antibody 9.2.27 (13) (a gift of the Biological Response Modifiers Program, National Cancer Institute, Frederick, MD) (100% of the cells) and MHC-I and MHC-II molecules (100 and 30%, respectively). HLA-A types of this cell line are HLA-A1, A2, and identical to the patient's PBL- and EB virus-transformed B lymphoblasts.

Hapten Modification

Autologous B lymphoblastoid cells and melanoma cells were modified with DNP by the previously described method (14). The DNP modification was confirmed by flow cytometry with a mouse monoclonal anti-DNP antibody (SPE-7; Sigma Chemical Co., St. Louis, MO). After DNP modification, cells were fixed with 70% ethanol on ice for 10 min and then stained with anti-DNP antibody, followed by sheep anti-mouse immunoglobulin antibody conjugated to FITC (Sigma). By this assay, 100% of the cells were shown to be modified with DNP.

Peptide Extraction

Peptides were extracted from MHC molecules of DNP-Mel, unmodified melanoma cells (MEL), DNP-modified B lymphoblastoid cells (DNP-Ly), and unmodified B lymphoblastoid cells (Ly) by the modified method of Rötzschke *et al.* [15]. In brief, 10^9 cells were suspended in 0.1% trifluoroacetic acid (TFA; pH 2.2) and then in 1% TFA, disrupted with a homogenizer and sonicator, and stirred at 4°C for 30 min. Insoluble fractions were removed by centrifugation at 141,000*g* for 30 min and samples were then lyophilized to remove the organic solvent. The released peptides were then reconstituted in 0.1% TFA and 5% acetonitrile and separated from proteins by using a Centricon-10 (Amicon, Bedford, MA) ultrafiltration devices (10 kDa cutoff) and fractionated by using reverse-phase HPLC on a Vydac C4 column (150 × 4.6 mm; 5 μm) (The Nest Group, Southborough, MA) with a Hewlett Packard 1050 HPLC system. HPLC solvents consisted of Buffer A (0.1% TFA, 5% acetonitrile, and 95 % H_2O) and Buffer B (0.1% TFA, 99.9% acetonitrile). Gradients consisted of the following linear step intervals; 0 to 5 min, 100% A; 5 to 45 min, 25% B (in A); 45 to 55 min, 50% B (in A); and 55 to 60 min, 100% B. Injection volume was 150 μl and flow rate was 1 ml/min. Absorbance was measured at 214 nm. Fractions were collected and dried by lyophilization.

Cytokine Production to Peptides

T cell responses to eluted peptides were measured by IFN-γ production. Each fraction of lyophilized peptides was reconstituted in 200 μl of phosphate-buffered saline solution. EB virus-transformed autologous Ly were inactivated by mitomycin C and plated into a 96-well round-bottom plate at 2×10^4 in 50 μl of lymphocyte culture medium. Ten microliters of peptide was added into each well and incubated for 1 hr at 37°C. After

this incubation period, T cells were added into each well at 10^5/well in 100 μl of culture medium and incubated for 18 hr, and then supernatants were collected for IFN-γ assay. IFN-γ production to autologous B lymphoblasts that had not been peptide loaded was also measured as background activity. For batch analysis, 5 μl of peptides was collected from each of 10 fractions and 25 μl of the combined peptide fractions was added to each well.

IFN-γ Assay

The concentration of IFN-γ in supernatants was measured by a commercially available ELISA kit (Endogen, Boston, MA; sensitivity, 5 pg/ml). The specific reaction to each peptide fraction was defined as IFN-γ (sample)-IFN-γ (background).

Spectrometric Analysis of DNP-Modified Peptides

DNP modification of the peptides was determined by analytical HPLC on a Vydac C18 column (25 × 4.6 mm; 5 μm). Two HPLC solvents, Buffer A (0.1% TFA, 99.9% H_2O) and Buffer B (0.1% TFA, 99.9% acetonitrile), were used in programmed gradient of 5–25% Buffer B over 40 min (0.5%/min). The fractionated peptides were injected into the analytical HPLC system at flow rate of 1 ml/min and absorbance was measured by spectrometer with wavelength of 200–380 nm. The eluted peptides were judged as DNP modified if a peak at 330–360 nm was detected (16).

RESULTS

T Cell Responses to DNP-Modified Autologous Melanoma Peptides

MHC-associated peptides eluted from DNP-Mel were loaded onto B lymphoblastoid cells and tested for ability to stimulate a T cell line responsive to intact DNP-Mel. As shown in Fig. 1, the T cells predominantly responded to a single peptide fraction 15 with a lower response to the adjacent peptide fraction 14.

Comparison of Peptides Eluted from Melanoma Cells and B Lymphoblasts

Based on the result obtained above, we further investigated the difference in T cell responses to DNP-Mel and unmodified Mel peptides and to DNP-Ly and unmodified Ly peptides. A new batch of peptides was prepared by growing 10^9 cells from autologous melanoma cell line (Mel) and EB virus-transformed B lymphoblasts. The cells were modified with DNP or left unmodified. Peptides were eluted from all four cell types. Initially, HPLC fractions were pooled into groups of 10. As shown in Fig. 2, the T cells only responded to the pool containing peptide fractions 11–20 from DNP-Mel. They did not respond to other peptides pools from DNP-Ly, Ly, or unmodified MEL.

Next, individual peptide fractions of pools 11–20 from DNP-Mel, unmodified Mel, and DNP-Ly or unmodified Ly were loaded onto autologous B lymphoblasts and tested for IFN-γ production by DNP-specific T cells. As shown in Fig. 3, the T cells responded to the DNP-Mel peptide fraction 18 and, to a lesser extent, to peptide fraction 17. The difference between this result and the result obtained in the first DNP-Mel peptide batch (Fig. 1) is likely to be due to slight variability in HPLC conditions. Peptide fractions 17 and 18 eluted from DNP-Ly were also stimulatory, although the response to fraction 18 from DNP-Mel was much greater than that from DNP-Ly. No significant response was evoked by any of the fractions of unmodified autologous cell peptides. The T cells also proliferated after stimulation with DNP-Mel fractions 17 and 18 (stimulation index, 2.4 and 3.9, respectively) and DNP-Ly fraction 17 (stimulation index, 2.0) but did not respond to other fractions by proliferation (data are not shown).

Demonstration That Stimulatory Peptides Are DNP Modified

To determine whether peptides in the stimulatory fractions were DNP modified, we analyzed selected fractions by spectroscopy at wavelengths of 200–380 nm using shallow gradients as described under Materials and Methods. Individual peptide fractions 11 to 20 from DNP-Mel were examined. As shown in Figs. 4b and 4c, peptides in fraction 18 from DNP-Mel eluted at 6.4 to 6.9 min exhibited absorption at 330 nm, indicating that they were DNP modified (16). In contrast, none of other nine peptide fractions from DNP-Mel elicited peaks at 330–360 nm. Representative results for one of these negative peptide fraction 11 are shown in Figs. 4e and 4f. A 330-nm peak was also detectable in peptide fraction 17 from DNP-Ly (data not shown).

The spectroscopy data were confirmed by blocking experiments with anti-DNP antibody. As shown in Fig. 5, IFN-γ production by DNP-specific T cells stimulated by DNP-Mel fraction 18 was almost completely blocked by anti-DNP antibody.

DISCUSSION

This study demonstrates that immunization with autologous melanoma cells modified with the hapten DNP induces a T cell response against hapten-modified, MHC-associated peptides. To our knowledge, this is the first demonstration of a T cell response to hapten-modified, MHC-associated peptides in the human system. The results of this study extend our previous find-

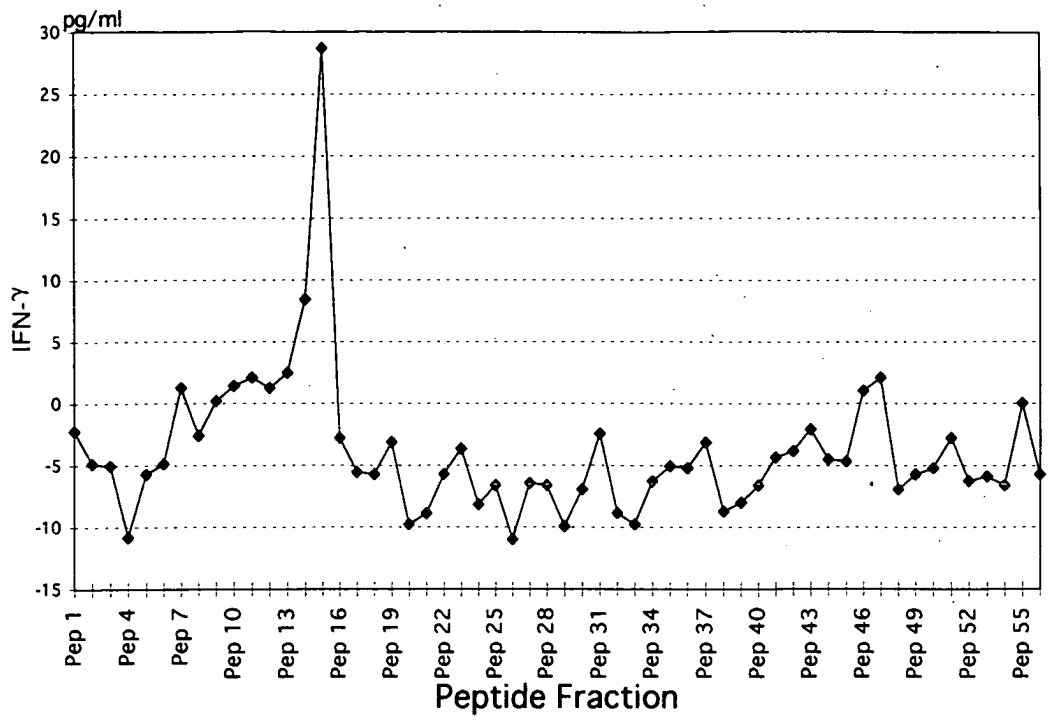

FIG. 1. T cell responses to HPLC fractions of DNP-Mel peptides. HPLC fractions of peptides were collected from DNP-modified, autologous melanoma cells. Each fraction was loaded onto autologous B lymphoblasts and then incubated with DNP-specific T cells. IFN-γ production of the T cells to B lymphoblasts without peptides (mean, 18.3 pg/ml) was subtracted from each test point as background.

ings of human T cell response to hapten-modified autologous cells (12).

Our findings are consistent with observations reported in animal experiments (17–20). For example, von Bonin *et al.* (17, 19) reported that trinitrophenyl (TNP)-specific T cell responses are directed to peptides anchored to the MHC molecules, not to hapten-modified MHC molecules themselves. They demonstrated that: (i) TNP modification of intact cells leads to the production of TNP-modified, MHC-associated peptides, (ii) such TNP-modified, MHC-associated self-peptides form antigenic epitopes for TNP-specific T cells; and (iii) TNP is a critical element for the T cell response.

In their experiments, mice appear to produce two types of T cell responses to hapten-modified, MHC-associated peptides. The first type is *sequence independent:* TNP-specific T cell clones recognize a variety of TNP-modified peptides provided that TNP-lysine is located at position 4 and appropriate anchoring side chains for the MHC groove are present (18). It is speculated that TNP may stimulate $J\beta/V\beta$-joining regions of T cell receptors, preserving the variability of TCR-α and -β regions (21, 22). The flexibility in the rest of the peptide sequence eases induction of hapten-related immunological responses since the chance that T cell receptors will encounter TNP-modified immunogenic epitopes increases dramatically.

The second type of T cell recognition of hapten-modified cells is *sequence dependent:* a minor fraction of TNP-specific T cell clones recognize only certain sequences of TNP-modified peptides. In this case, designer peptides have revealed a complex antigenic determinant comprised of TNP-lysine in position in 7 and unmodified amino acids in positions 3 and 4 (18). Interestingly, these T cell clones also recognize unmodified peptides; i.e., there was associative recognition of *unmodified* peptides by T cell clones generated by immunization with hapten-modified peptides. A similar observation has been made in another murine system: T cell hybridomas responsive to hen egg lyzozyme (HEL) modified with the hapten, phosphorylcholine recognized unhaptenized HEL as well as haptenized HEL (23).

This phenomenon might explain our clinical findings that hapten-modified autologous melanoma cell vaccine induces inflammation in remote metastases (6) and that the inflammatory response includes T cell clones with unique structures that recognize unmodified melanoma cells (11). This paper shows that vaccination with hapten-modified autologous melanoma cells induces T cells which react with hapten-modified tumor peptides on the MHC of melanoma cells. A minor fraction of these T cells may associatively recognize unmodified tumor peptides and consequently be attracted to the metastatic sites. Clonal analysis of the

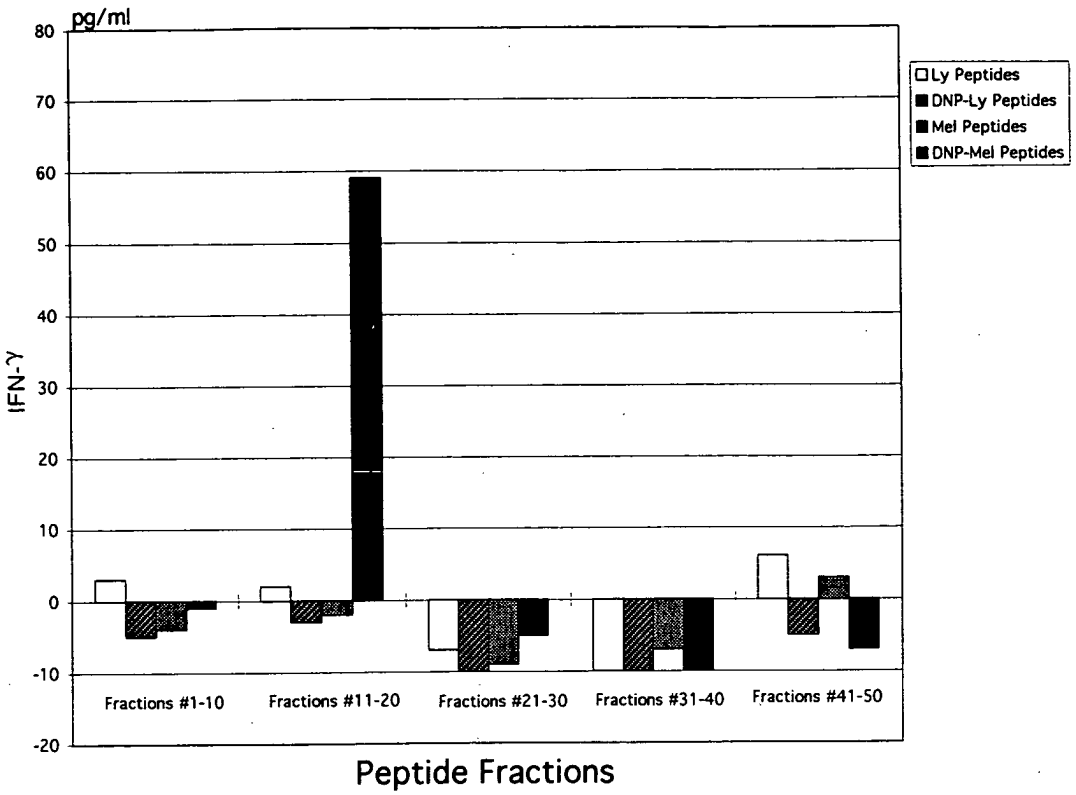

FIG. 2. Batch analysis of peptide fractions from DNP-modified and unmodified Mel and Ly. A new HPLC fraction of peptides was prepared from the autologous melanoma cell lines and from autologous EB virus-transformed B lymphoblasts. The cells were either modified or unmodifed with DNP and then small peptides were eluted from the MHC and fractionated by HPLC. Ten fractions of the peptides from each cell type were collected into one batch and tested for ability to stimulate IFN-γ production by T cells. Ly, DNP-Ly: DNP-unmodified (Ly) or modified (DNP-Ly), EB virus-transformed autologous B cells, Mel, DNP-Mel: DNP-unmodified (Mel) or modified (DNP-Mel), autologous melanoma cell line.

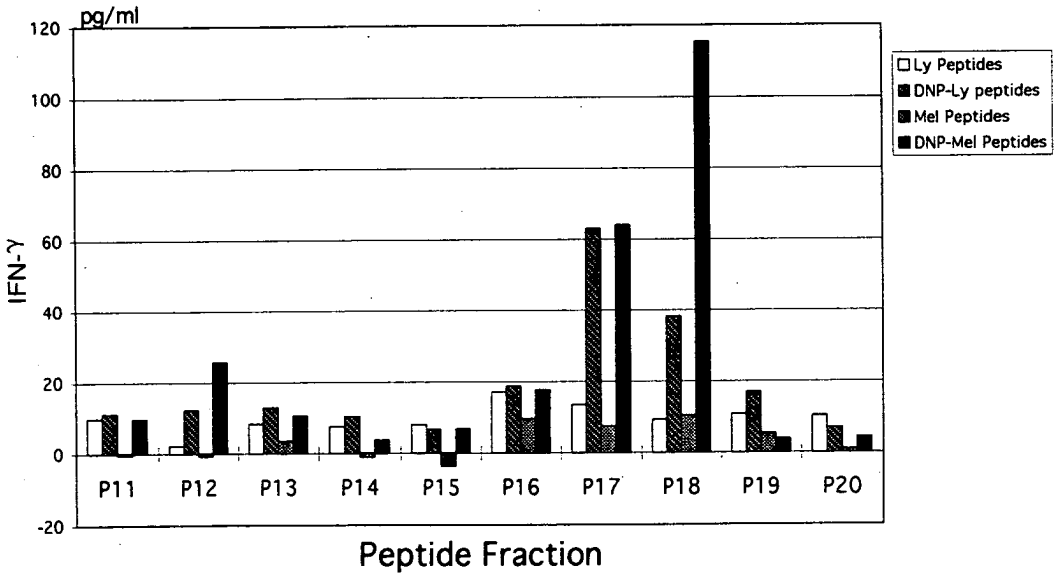

FIG. 3. T cell response to individual peptide fractions from batches 11–20. Peptides were prepared as described in the legend to Fig. 2. IFN-γ production of the T cells to B lymphoblasts without peptides (mean, 41.5 pg/ml) was subtracted from each test point as background.

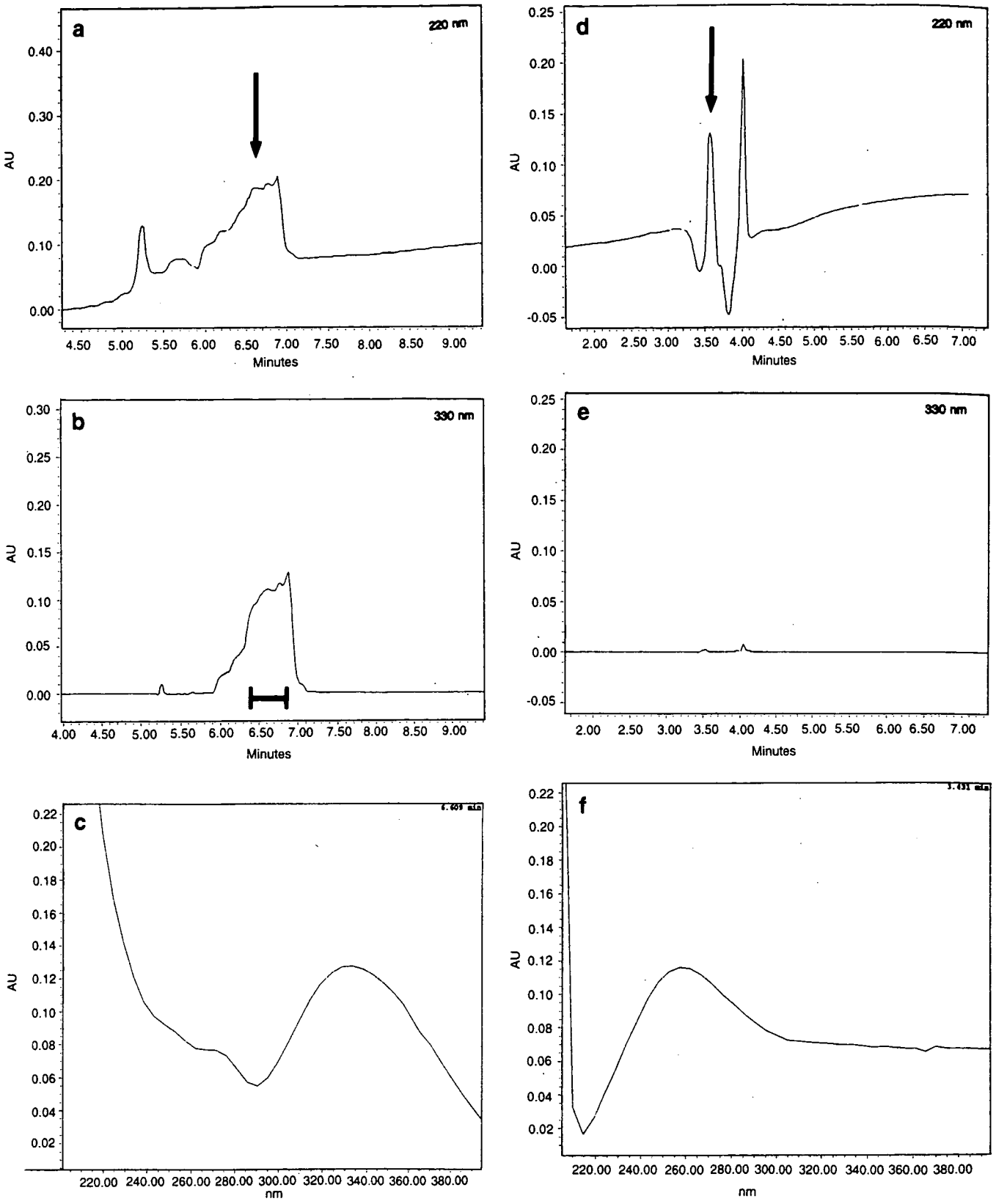

FIG. 4. Spectrometric analyses of DNP-modified melanoma peptide fractions. Individual DNP-Mel fractions 11–20 were analyzed by HPLC with absorbance at 200–380 nm. The results of fraction 11, which did not stimulate DNP-reactive T cells, and 18, which did stimulate DNP-reactive T cells, are shown. (a) Absorbance of peptide fraction 18 measured at 220 nm. (b) Absorbance of peptides fraction 18 measured at 330 nm. (c) Absorbance of representative peptide peak (indicated by arrow in a) at wavelengths of 200–380 nm. The peptides retained between 6.4 to 6.9 min (shown with a bar in b) elicited a peak at 330 nm. (d) Absorbance of peptide fraction 11 measured at 220 nm. (e) Absorbance of peptides fraction 11 measured at 330 nm (no peak seen). (f) Absorbance of representative peptide peak (indicated by arrow in d) at wavelengths of 200–380 nm. No 330-nm peak is present.

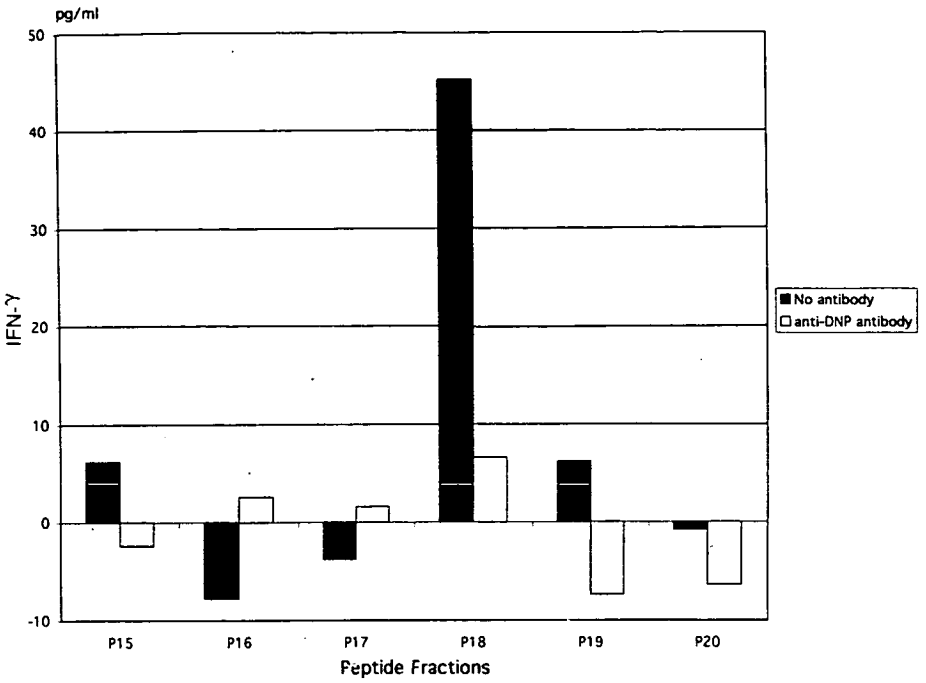

FIG. 5. Blocking of T cell response to DNP-Mel fractions by anti-DNP antibody. Autologous B lymphoblasts loaded with a fraction of DNP-Mel peptides were incubated with anti-DNP antibody for 1 hr prior to the addition of DNP-specific T cells. IFN-γ production by the T cells were measured after an 18-hr incubation.

DNP-specific T cell line and sequence analysis of the stimulatory fraction of DNP-Mel will be required to test this hypothesis.

It remains to be answered why our DNP-specific T cell line responded predominantly to a single fraction of peptides extracted from the DNP-modified autologous melanoma cell line. The spectrometric analyses of the stimulatory DNP-Mel peptide fraction 18 suggest that multiple peptides in this fraction were modified with DNP. It is possible that different DNP-modified melanoma peptides with different structures, but with lysine in the same position, are collected into a single HPLC fraction. In fact, von Bonin *et al.* (19) demonstrated that TNP-modified peptides eluted from TNBS modified mouse splenic cells are collected in contiguous HPLC fractions and strong cytotoxic T cell responses are elicited to target cells loaded with these fractions of hapten-modified peptides. In contrast, only a weak response was induced by a noncontiguous fraction. It is also possible that in our system additional fractions of DNP-modified peptides were present but below the threshold of detection by our spectrometric analysis.

Identification and sequencing of the DNP-modified peptides that mediate T cell responses in our system would yield both practical and heuristic benefits. Such peptides might be able to serve as effective melanoma vaccines, perhaps obviating the need to prepare a different cellular vaccine for each patient. Furthermore, explication of the structure of the sequence-dependent peptides could lead to the structure of the unmodified peptides with which they are cross-recognized. In turn, this could result in the discovery of new, immunogenic melanoma antigens. Thus attempts at elucidating peptide structures, although tedious and technically daunting, appear to be justified.

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